UNIVERSIDADE FEDERAL DO CEARÁ
CENTRO DE CIÊNCIAS
DEPARTAMENTO DE BIOQUÍMICA E BIOLOGIA MOLECULAR
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOQUÍMICA

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SYSTEMATIC REVIEW OF THE CHLOROPLASTIC REDOX NETWORK AND NEW INSIGHTS INTO THE FUNCTION OF Trx *h*2 in *Arabidopsis thaliana L*.

FORTALEZA
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Dissertação apresentada ao Programa de Pós-Graduação em Bioquímica da Universidade Federal do Ceará, como requisito parcial para a obtenção do título de Mestre em Bioquímica. Área de concentração: Bioquímica Vegetal

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FORTALEZA

2018

Dados Internacionais de Catalogação na Publicação
Universidade Federal do Ceará
Biblioteca Universitária
Gerada automaticamente pelo módulo Catalog, mediante os dados fornecidos pelo(a) autor(a)

Souza, Paulo Vinicius Leite de.
Systematic review of the chloroplastic redox network and new insights into the function of Trx h2 in Arabidopsis thaliana. L / Paulo Vinicius Leite de Souza. – 2018.
117 f. : il. color.

Dissertação (mestrado) – Universidade Federal do Ceará, Centro de Ciências, Programa de Pós-Graduação em Bioquímica, Fortaleza, 2018.
Orientação: Prof. Dr. Danilo de Menezes Daloso.

1. Thioredoxin . 2. Redox metabolism . 3. Thioredoxin h2. I. Título.

CDD 572

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Aprovada em: 12/07/2018.

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AGRADECIMENTOS

Primeiramente gostaria de agradecer a Deus. Imensamente agradecer a minha família por sempre me apoiar em todas minhas decisões. Em especial agradeço aos meus pais, Lauriane e Julio por tudo que fizeram ao longo dos anos, mesmo nos momentos mais difícil que passamos juntos sempre superamos, e entre todos os sentimentos que já passamos o único que nunca se alterou foi o amor que sentimos. Claro gostaria de agradecer aos meus irmãos, Gabriela e Julio Filho, por toda confiança e suporte que depositaram em mim. Em especial gostaria de agradecer minha vó que ao longo desses anos se tornou uma segunda mãe.

Imensamente agradeço a orientação dada pelo Professor e Doutor Danilo de Menezes Daloso, que tive ao longo destes dois anos. Graças a ele tive o suporte para que me tornasse um profissional mais capacidade, fornecendo sempre uma excelente orientação, se mostrando dispostos para sugestões e sendo sempre paciente. Assim se tornando um espelho de profissional que um dia espero alcançar. Muito obrigado por tudo!

Em especial, gostaria de agradecer ao Professor Joaquim Albenísio Gomes da Silveira, por me aceitar em seu laboratório. Fornecendo sempre apoio, conhecimento e assim se tornando um exemplo de profissional para mim.

Agradeço aos membros do Labplant; Pos-Doctor Fabricio, Ana Karla e Leticia por fornecerem sempre conhecimento, e sempre estarem dispostos. Também agradeço a todos integrantes do Lab plant (Valéria, Vicente, Rikaely, Yugo, William, Cristiano, Markos, Adilton, Eliezer, Bruno, Silvio, Raissa) que se tornaram uma segunda família ao longo desses anos.

Agradeço a minha namorada (Kassia Lia) por estar sempre presente e paciente.

Agradeço a todos os meus amigos que fizeram parte da minha vida (Lucas, Romulo, Pablo, Iasmim, Juan, Pedro, Diego, Ilana, Patrick, Erica, Amanda, Carol, Ramon, Renata) e por sempre acreditarem no meu potencial.

Agradeço aos Professores Murilo, Fabricio e André por terem aceitado o convite para participarem da minha banca de defesa.

O presente trabalho foi feito com o apoio financeiro da Coordenação de aperfeiçoamento de pessoal do nível superior- Brasil (CAPES).

ABSTRACT

As sessile organisms, plants are heavily influenced by the environmental condition. To maintain homeostasis, plants need precise and refined metabolism, fast and accurate, in response to environmental changes. In this context, redox metabolism is essential to maintain homeostasis by programmed gene expression and post-translational modifications through redox regulation mediated by thioredoxin (Trx). Trxs are important enzymes in the control of redox metabolism. Trxs are mainly located in chloroplasts, cytosol and mitochondria, with a greater predominance in chloroplasts. In this sense, the Trxs of the chloroplast are the most studied, being responsible for the regulation of Calvin-Benson cycle proteins, starch metabolism, chlorophyll biosynthesis and ATP synthesis. However, our understanding of the role of the redox Trxs of these proteins in other cellular compartments remains unclear. This is due to the fact that chloroplast Trxs are often studied in isolation, making it difficult to understand the redox system working in harmony while maintaining cellular homeostasis. By contrast of the chloroplastic Trx network, the role of mitochondrial Trxs and the function of Trx *h* isoforms, which are beyond the chloroplast, remain unclear, especially in the case of the Trx *h2* isoform. This isoform is located at both cytosol and mitochondria. Trx *h2* was recently proposed to regulate the TCA cycle succinate dehydrogenase and fumarase enzymes plus alternative oxidase of the electron transport rate. However, no studies have confirmed whether this also occur *in vivo*, which is especially due the lack of *trxh2* mutant characterization. Here, we thus characterized *trxh2* mutant lines aiming to provide information regarding the role of this isoform for plant growth and leaf metabolism. This thesis is divided in two chapters. In the first, we provided a precise and refine review of chloroplastic redox metabolism, evidencing its main characters and the importance of redox metabolism on cellular homeostasis. After that, we described the results from experimental characterization of *trxh2* mutant lines**.**

Keywords: Thioredoxin. Redox Metabolism. Thioredoxin h2.

RESUMO

Como organismos sésseis, as plantas sofrem grande influência da condição ambiental. Para manter a homeostase, as plantas precisam de um metabolismo preciso e refinado, rápido e preciso, em resposta a mudanças ambientais. Nesse contexto, o metabolismo redox é essencial para manter a homeostase pela expressão genética programada e modificações pós-tradução através regulação redox mediada pelas tiorredoxinas (Trx). Trxs são enzimas importantes no controle do metabolismo redox. Trxs sãos localizada principalmente nos cloroplastos, citosol e mitocôndrias, com maior predominância nos cloroplastos. Neste sentido, as Trxs do cloroplasto são as mais estudadas, sendo responsáveis pela regulação de proteínas do ciclo de Calvin-Benson, metabolismo de amido, biossíntese de clorofila e síntese de ATP. No entanto, nossa compreensão sobre a o papel das Trxs redox destas proteínas em outros compartimentos celulares permanece sem muitos conhecimentos. Isso devido pelo fato das Trxs cloroplástica serem frequentemente estudados isoladamente, dificultando a compreensão do sistema redox trabalhando em harmonia mantendo a homeostase celular. Em contraste com a rede Trx cloroplástica, o papel das Trxs mitocondriais e a função das isoformas Trxh, que estão além do cloroplasto, permanecem incertas, especialmente no caso da isoformas Trx h2. Esta isoforma está localizada tanto no citosol como nas mitocôndrias. Recentemente, o Trx h2 foi proposto para regular as enzimas succinato desidrogenase e fumarase do ciclo TCA, além da oxidase alternativa da cadeia transporte de elétrons. No entanto, nenhum estudo confirmou se isso também ocorre in vivo, o que é especial marcado pela à falta de caracterização do mutante trxh2. Assim, caracterizamos linhagens de mutantes trxh2 com o objetivo de fornecer informações sobre o papel desta isoforma no crescimento das plantas e no metabolismo foliar. Esta dissertação é dividida em dois capítulos. No primeiro, fornecemos uma revisão precisa e refinada do metabolismo redox, do cloroplasto evidenciando seus principais personagens e a importância do metabolismo redox na homeostase celular. Em seguida, descrevemos os resultados da caracterização experimental das linhagens trxh2 mutante.

Palavras chaves: Thioredoxina. Metabolismo Redox. Tiorredoxina h2.

FIGURE LIST

TABLE LIST

SUMMARY

1 INTRODUCTION

By contrast to animals, plants are sessile organisms. As such they require a higher phenotypic plasticity in the face of prevailing environmental changes. They accomplish this, in part, by the increased number of gene duplications and thereby protein isoforms found in plants, which are distributed in the three genomes located at the nucleus, chloroplasts, and mitochondria (ARABIDOPSIS GENOME INITIATIVE, 2000). These genomes encode proteins localized in different plant cell organelles which interact with one another following endogenous and environmental signals and induce transcriptional and post translational alterations, ultimately regulating metabolic fluxes and their associated physiological response (GEIGENBERGER; FERNIE, 2014a). Many enzymes of plant cell metabolism are subjected to a post translational regulation including acetylation, carboxylation, sumoylation, phosphorylation, and redox regulation (FRISO; VAN WIJK, 2015). Plants possess an unprecedentedly complex redox regulation system, in which the presence of different redoxins (thio-, gluta-, peroxi-, sulfoxi-, ferro-) and peroxidases (ascorbate-, glutathione-) are of pivotal importance to maintain growth and development (FOYER, C. H.; NOCTOR, 2011; GEIGENBERGER e colab., 2017; REICHHELD, Jean Philippe e colab., 2010). Despite their important role in the redox regulation of metabolism, it has been shown that several of these enzymes are not essential for plants, meaning that plants individually lacking some of these proteins present no apparent phenotype and can survive even under stress conditions. This suggests that the components of the redox network may be able to compensate one another in order to maintain homeostasis. However, precise mechanistic details of the complementarity between different components of the redox system and how they interact with one another remains elusive -especially so for those components associated with the chloroplast.

Life on earth depends on the process of photosynthesis which in most species takes place in the chloroplast. This organelle contains the most complex redox system found in plants (BUCHANAN, 2016b). The chloroplastic redox system is involved in the regulation of different important physiological processes including the light-dependent redox activation of Calvin-Benson cycle enzymes and the avoidance of reactive oxygen species (ROS) overaccumulation (BUCHANAN, 2016a; NOCTOR e colab., 2018). Thus, the chloroplastic redox system contributes not only to activate the process of CO_2 fixation but also to avoid oxidative stress caused by ROS overaccumulation. It seems likely that CO_2 fixation is mainly regulated by the

chloroplastic thioredoxin system, which is composed by different thioredoxins (TRX) and TRX-reductases such as ferredoxin reductase (FTR) and NADPH-dependent thioredoxin reductase C (NTRC) (Table 1; Figure 1). The TRX system is capable to regulate the redox status of thiols by reversibly controlling the formation or degradation of the disulfide bridge formed between two Cys residues in the target protein. This leads to alteration in the structure of the protein that leads to loss or gain of function (MEYER e colab., 2009). Given the high number of proteins that are redox regulated, it is reasonable to conclude that the redox status of Cys residues is of pivotal importance for the regulation of metabolism. Furthermore, recent evidence suggests that enzymes of, or associated to, the tricarboxylic acid (TCA) cycle that are redox regulated contain at least two Cys residues conserved between microorganisms, animals, and plants (DALOSO e colab., 2015b). This suggests that redox regulation of metabolism is, in itself, a conserved mechanism, although it assumes a higher degree of complexity in plants given the presence of additional isoforms and the need to cope with constant adverse environmental conditions.

A wide range of different environmental stress conditions leads to oxidative stress within plant cells, which is mainly due the overaccumulation of different ROS such as singlet oxygen ($^1O_2$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2). Although ROS accumulation can be due to the reactions occurring in other compartments such as mitochondria, cytosol and peroxisomes, the chloroplast has been documented as one of the most important sources of ROS in plants (MITTLER, 2017). Given its higher stability among the ROS, H_2O_2 has been suggested to be the key molecule involved in retrograde signalling and in the oxidation of thiols of redox-regulated proteins (Noctor *et al.*, 2017). H_2O_2 assumes therefore a pivotal importance in the interorganellar communication and in the deactivation of redox regulated enzymes, being a counter-point of the TRX system (FARNESE e colab., 2016; PESARESI e colab., 2007). However, it is important to highlight that plants must avoid overaccumulation of H_2O_2 and other ROS given the harmful potential of these molecules. For this, plants possess innumerous antioxidant enzymes such as ascorbate peroxidase (APX), glutathione peroxidase (GPX), superoxide dismutase (SOD), and peroxiredoxin (PRX) (Table 1) that are not only capable of removing the excess of ROS but also regulate the balance of reduced and oxidized forms of ascorbate (ASC and DHA) and glutathione (GSH and GSSG) (Figure 1).

It is interesting to note that whilst animal GPXs exclusively use GSH as electron donor (PASSAIA; MARGIS-PINHEIRO, 2015), plant GPXs display a considerably higher affinity to

use TRXs as electron donor (HERBETTE e colab., 2002; IQBAL e colab., 2006; JUNG e colab., 2002). Additionally, different PRXs, 2-Cys PRXs and methionine sulfoxide reductase (MSR) have also been demonstrated to be regulated by TRX (Figure 1). This indicates that ROS metabolism, ROS scavenging and the thioredoxin systems are tightly interconnected. However, unfortunately these systems have largely been studied and reviewed separately. Furthermore, despite the advances obtained in unraveling key points of metabolic regulation by adopting systems biology approaches; they have surprisingly not been often adopted in the study of plant redox metabolism. Thus, an integrative view of these systems is urgently needed to unravel the connection and the complementarity between these systems in plants. Here we first briefly review the function and the knowledge accumulated of each component of the chloroplastic redox system and then discuss how these systems work co-operatively to maintain the redox status of the cell under both normal and adverse conditions. The outstanding questions that should be addressed in this field in order to improve our understanding on how the plant redox system works and regulates the photosynthetic process are highlighted throughout the text. We finally provide a perspective concerning how the characterization of plants lacking multiple components of the chloroplastic redox network and the adoption of systems biology approaches can be used to reach these goals.

2 FUNCTION AND COMPENSATORY MECHANISMS AMONG THE COMPONENTS OF THE CHLOROPLASTIC REDOX NETWORK

2.1. The versatility of the chloroplastic thioredoxin system

TRXs comprise an ancient family of proteins found in both prokaryote and eukaryote organisms that regulate the redox status of target proteins (Figure 2) (BUCHANAN e colab., 2012). TRXs contain a redox-active dithiol cysteine residue which allows these small polypeptides to catalyze the reduction of disulfide bonds, regulating the function and structure of target proteins (MEYER e colab., 2012b). Chloroplastic TRXs are mostly reduced by FTR and to a lesser extent by NTRC (Figure 2) (LEMAIRE e colab., 2007; TAN e colab., 2010). Unlike the situation observed in other organisms, plant TRXs are encoded by a large gene family. For instance, the model plant *Arabidopsis thaliana* L. contains more than 20 TRX isoforms of which TRXs *f1-2, m1-4, x, y1-2,* and *z* are located at the chloroplast, TRX *o1-2* in the mitochondrion,

and the proteins of the TRX *h* family (TRX *h1-9*) are distributed in cytosol, nucleus, mitochondria, plasma membrane and endoplasmic reticulum (BELIN e colab., 2015; DELORME-HINOUX e colab., 2016). In addition to the canonical chloroplastic TRXs, additional TRX-like proteins such as the chloroplast drought-induced stress protein of 32 kDa (CDSP32) (BROIN; REY, 2003), four ACHT proteins (DANGOOR e colab., 2009) and other TRX-like proteins are also found in the chloroplasts (MEYER e colab., 2009). Given their wide distribution within plant cells, TRXs have been involved in the regulation of several key processes over the entire plant life cycle, especially those associated with chloroplast function (GEIGENBERGER e colab., 2017).

It has been shown that TRXs *m* and *f* regulate several proteins of the Calvin-Benson cycle (CBC), chlorophyll biosynthesis, starch synthesis, oxidative pentose phosphate pathway, malate metabolism, and ATP synthesis (COLLIN e colab., 2003; COURTEILLE e colab., 2013; LAUGIER e colab., 2013; OKEGAWA; MOTOHASHI, 2015; THORMÄHLEN e colab., 2013; WANG, P. e colab., 2013; YOSHIDA e colab., 2015). By contrast, TRXs *x*, *y*, and *z* are less studied and their function has been limited to the regulation of stress-related proteins such as 2-Cys PRXs, thiol-peroxidases and methionine sulfoxide reductase (MSR) (Geigenberger *et al*., 2017). Whilst the TRX-target proteins were revealed by proteomic studies, the physiological function of TRXs has been demonstrated mainly by the characterization of TRX mutants. For instance, the characterization of *trxm* mutants has revealed the involvement of the TRXs *m3* and *m4* in the regulation of cyclic electron transport (BENITEZ-ALFONSO e colab., 2009; COURTEILLE e colab., 2013). Furthermore, although single or combined mutation in TRXs *m1* and *m2* revealed no changes in photosynthetic parameters under normal growth conditions (LAUGIER e colab., 2013; OKEGAWA; MOTOHASHI, 2015), these proteins proved to be important under fluctuating light and high light conditions (THORMÄHLEN e colab., 2017), most probably due to their capability to regulate the chloroplastic malate valve and ATP synthesis (CARRILLO e colab., 2016; NIKKANEN e colab., 2016; THORMÄHLEN e colab., 2017; WOLOSIUK e colab., 1977; YOSHIDA; HISABORI, 2016b). Similarly to *trxm1* and *trxm2* single mutants, the single *trxf1* mutant and the double *trxf1f2* mutant showed no apparent phenotype despite a deficiency and a delay in light activation in the CBC-fructose-1,6-biphosphatase (FBPase) enzyme and in the starch synthesis-related enzyme ADP glucose pyrophosphorylase (AGPase) (NARANJO e colab., 2016; THORMÄHLEN e colab., 2013;

YOSHIDA e colab., 2015). More importantly, triple *trxm1-m2-m4* mutant plants displayed a visible phenotype alteration being characterized by a pale-green color and a 50% reduction in their photosynthetic rate (WANG, Jia; VANLERBERGHE, 2013), highlighting the compensatory role between *m* type TRXs. Taken together, these studies were essential in demonstrating the role of these TRXs *in vivo* and suggest that TRXs *f* and *m* are key regulators of the carbon assimilation photosynthetic pathway. Furthermore, the fact that the observed phenotype is more severe when more than one TRX is mutated demonstrates the capacity of the chloroplastic TRX system in adjusting its main actors in order to maintain chloroplastic redox homeostasis. Beyond this cooperation between chloroplastic TRXs, it is known that both chloroplastic TRX reductase systems also work co-operatively (YOSHIDA; HISABORI, 2016b). Thus, it seems likely that the NTRC-TRX and Fdx-TRX systems form an interconnected redox network which enables plants to respond according to the light condition (PÉREZ-RUIZ e colab., 2017).

a. The critical but non-essential role of the NADPH-dependent thioredoxin reductase C in chloroplasts

Chloroplastic TRXs are mainly reduced by FTR and to a minor extent by NTRC. TRXs from other compartments are mainly reduced by NTRA and NTRB, although a compensatory role of the NADPH/GR/GSH/GRXs system in reducing TRXs has also been observed (REICHHELD, Jean-Philippe e colab., 2007). NTRC is exclusively found in plastids whilst NTRA and NTRB are both simultaneously located in the cytosol and mitochondria, which potentiates redundancy amongst these proteins and explains the absence of phenotype apparent in the *ntra* and *ntrb* single mutants (REICHHELD, Jean-Philippe e colab., 2007; REICHHELD, Jean Philippe e colab., 2005). Whilst NTRA and NTRB present only TRX reductase activity, NTRC contain an unusual TRX domain which allows this protein to act simultaneously as TRX and TRX reductase. Firstly described in *Oryza sativa* (SERRATO e colab., 2004), NTRC was initially pointed out as an alternative protein involved with redox regulation of chloroplastic metabolism (PEREZ-RUIZ, 2006). However, biochemical studies and reverse genetic characterization of rice and Arabidopsis *ntrc* mutants demonstrated the importance of this protein and led to its inclusion as a major player in chloroplastic redox metabolism (PEREZ-RUIZ, 2006; SERRATO e colab., 2004; THORMÄHLEN e colab., 2015). The Arabidopsis *ntrc* knockout mutant presented severely impaired photosynthesis rate and reduced growth rate (PEREZ-RUIZ,

2006; SERRATO e colab., 2004; THORMÄHLEN e colab., 2015). However, despite of its extreme importance for plant growth, it is noteworthy that this system is not essential given that the single *ntrc* mutant is still viable. This viability is abolished in the *ftrb ntrc* double mutant (PÉREZ-RUIZ e colab., 2017; YOSHIDA; HISABORI, 2016b), indicating that FTR can compensate the role performed by NTRC and that a complete TRX reductase system is indispensable for plant growth and development.

The function of NTRC differs from that of the Fdx/TRX system, which fundamentally uses electrons from photosystem I (PSI), while NTRC uses NADPH as electron donor. The source of NADPH for NTRC can be from the photosynthetic electron transport chain in the light or the oxidative pentose phosphate pathway (OPPP) which also works under dark conditions (MONTRICHARD e colab., 2009). This implies that NTRC can also act in non-green tissues such as root amyloplasts (MICHALSKA e colab., 2009). In fact, the Arabidopsis *ntrc* mutant is hypersensitive to darkness (PÉREZ-RUIZ e colab., 2017). However, the phenotype of the *ntrc* mutant is more severe when plants were grown under a short day photoperiod or under fluctuating light conditions (THORMÄHLEN e colab., 2015, 2017). The function of NTRC during light exposure is mainly linked to the regulation of stromal enzymes that are involved in either the CBC or H_2O_2 detoxification (Perez-Ruiz *et al*., 2006). The NTR domain of NTRC is able to reduce TRXs such as *f1*, *m1*, *m3*, *x*, and *y1 in vitro* even when its TRX domain is inactivated (Yoshida and Hisabori, 2016). Furthermore, NTRC is involved in starch biosynthesis by activating AGPase (LEPISTÖ e colab., 2013; MICHALSKA e colab., 2009), ATP synthesis by activating the CF1γ subunit of ATP synthase (NIKKANEN e colab., 2016), and tetrapyrrole biosynthesis by activating two key enzymes of this pathway, the magnesium chelatase and magnesium protoporphyrin methyltransferase (PÉREZ-RUIZ e colab., 2014; RICHTER e colab., 2013).

Interestingly, the NTRC-mediated CF1γ activation seems to be independent of the TRXs *f1* and *f2* given that the *trxf1-f2* double mutant did not show impairment in light-dependent reduction of CF1γ (YOSHIDA e colab., 2015), despite the interaction observed between TRX *f1* and CF1γ *in vivo* (NIKKANEN e colab., 2016). It has been suggested that the NTRC-mediated CF1γ activation occurs mainly under low light condition whilst the FTR-TRX *f1* system is able to compensate NTRC under a high light environment (CARRILLO e colab., 2016; NIKKANEN e colab., 2016). Among the CBC enzymes, FBPase is the most clearly described enzyme that it is

regulated by NTRC *in vitro* and *in vivo* (THORMÄHLEN e colab., 2015). Beyond the deficiency in activating FBPase, which reduces the capacity of the plant to regenerate ribulose-1,5-bisphosphate in the CBC, the reduced growth phenotype of *ntrc* is also due to an impairment in photochemical quenching, as demonstrated by the lower values of the effective quantum yield of PSII (THORMÄHLEN e colab., 2015). As a consequence, the *ntrc* deficiency leads to decreased photosynthetic efficiency in different light growth conditions (CARRILLO e colab., 2016; NARANJO e colab., 2016; YOSHIDA; HISABORI, 2016b). On the other hand, the overexpression of NTRC and TRX *f1* leads to increased biomass accumulation and starch biosynthesis in tobacco (SANZ-BARRIO e colab., 2013). This highlights the importance of the chloroplast NTR/TRX system for plant growth. Furthermore, given the importance of enzymes of the antioxidant system for plant stress tolerance and based in the fact that several proteins of this system are redox regulated by TRXs, it seems reasonable to assume that the chloroplast NTR/TRX system may contribute to the plant stress acclimation by modulating the activity of antioxidant enzymes. In the next sections, we will briefly describe the functions of important enzymes of the antioxidant system such as PRXs, SRXs, APXs, GPXs, and other glutathione-related enzymes in plant cells and will also discuss how these proteins interact with FTR, NTRC and TRXs in the regulation of chloroplast metabolism.

b. The interplay between thioredoxins, peroxiredoxins, and sulfiredoxins in chloroplasts

Peroxiredoxins (PRXs) and sulfiredoxins (SRXs) are important components of the chloroplastic antioxidant defense system (DIETZ, Karl-Josef, 2011). SRX is conserved in eukaryotes yet absent in prokaryotes, with the exception of cyanobacteria (ROUHIER e colab., 2006). Like the TRXs, PRXs are also small proteins (with their apparent molecular masses ranging from 17 to 22 kDa) and are widely distributed within plant subcellular compartments (DIETZ, Karl Josef e colab., 2006; SEVILLA e colab., 2015). The 2-Cys PRXs are the most abundant PRX in plants. SRX and 2-Cys PRXs form an interconnected network with the TRX system, given that 2-Cys PRXs reduction depends on the activity of TRX and/or SRX. Moreover, evidence suggests that the presence of NTRC is important for 2-Cys PRX reduction (PUERTO-GALÁN e colab., 2015; SEVILLA e colab., 2015). The oxidation state of the Cys residues of 2-Cys PRXs determines whether the reduction is performed by SRX or by the NTR/TRX system

(Figure 1). This is based on the fact that one of the two Cys residues of 2-Cys PRX can be oxidized by H_2O_2 to form sulfenic acid (SOH) which can be subsequently further oxidized by H_2O_2 to form sulfinic acid (SO_2H). The overoxidized SO_2H is reduced by SRX to form SOH, which can then be reduced to SH by TRX (Biteau *et al.*, 2003; Jeong *et al.*, 2006; Puerto-Galán *et al.*, 2015). It has been proposed that the interconversion between reduced, oxidized and overoxidized status of 2-Cys PRX have an important role in the homeostasis of H_2O_2 content by acting as ROS sensors (LIEBTHAL e colab., 2017), including this enzyme as another component of the ROS-scavenging system. This idea is based on the fact that two molecules of H_2O_2 are consumed for the overoxidation of 2-Cys PRX. In this respect, SRX proteins are of pivotal importance to reduce the overoxidized form of the 2-Cys PRX, which can then be reduced by the TRX system, enabling the restart of the H_2O_2 consuming cycle (Figure 1).

The known function(s) of SRX have until recently been limited to specifically reduce sulfinic acid of 2-Cys PRXs, given that *srx* mutant present increased level of overoxidized 2-Cys PRX (PUERTO-GALÁN e colab., 2015) and based in the fact that SRX is not capable to reduce the same oxidized Cys residue in other PRXs or in glyceraldyhyde-3-phosphate dehydrogenase (HYUN e colab., 2005). However, interesting results from a recent 2-Cys PRX interactome study revealed that this protein interacts with several other chloroplastic proteins related to photosynthesis, carbon, nitrogen and sulfur metabolisms, antioxidant defense, and the TRX system (CERVEAU e colab., 2016). What remains unclear is how the PRX-SRX cycle interacts with the FTR/NTRC-TRX system (LIEBTHAL e colab., 2017). Furthermore, it was shown that 2-Cys PRX interacts with important enzymes of the antioxidant system such as SOD, GR and PRXQ, raising the question whether these proteins act in concert in the regulation of ROS homeostasis. In the next sections we review the role of other chloroplastic ROS-scavenging enzymes such as APX and GPX as well as the glutathione metabolism in the regulation of chloroplastic redox metabolism.

c. Chloroplastic APX and GPX: more than ROS scavenging enzymes

APXs and GPXs are other important enzymes of the antioxidant defence system involved in ROS scavenging in plant cells. APX and GPX isoforms are present in almost all plant cell compartments including cytosol, chloroplast, peroxisome and mitochondria (MARGIS e colab., 2008; TEIXEIRA e colab., 2006). Most plant species harbour two different chloroplastic APX

isoforms (chlAPX), one targeted to the stroma (sAPX) and the other to thylakoid membranes (tAPX) (Table 1) (TEIXEIRA e colab., 2006). The most remarkable evidence for the biological role presented by chlAPXs is provided by the characterization of plants lacking sAPX and/or tAPX (CAVERZAN e colab., 2014; DANNA, 2003; GIACOMELLI e colab., 2007; KANGASJÄRVI e colab., 2008; MARUTA e colab., 2010; MILLER e colab., 2007). Except in wheat (DANNA, 2003), plants deficient in both chlAPX isoforms are capable of tolerating excessive light (CAVERZAN e colab., 2014; GIACOMELLI e colab., 2007; KANGASJÄRVI e colab., 2008; MARUTA e colab., 2010). For instance, Arabidopsis mutants and rice antisense transgenic plants for both chlAPXs presented no phenotypic differences compared to WT plants even under high light conditions (Giacomelli *et al.*, 2007; Caverzan *et al.*, 2014). A similar study reported that tAPX is important in the first hours of high light exposure, but not in long term light exposure (MARUTA e colab., 2010). These results suggest that these proteins are not essential for plant stress responses but may rather be mainly related to the mediation of H_2O_2 retrograde signalling.

By contrast to cytosolic isoforms that actively work to prevent excessive ROS accumulation, chlAPX isoforms likely display an important role at the onset of photooxidative stress, controlling H_2O_2-induced retrograde signalling and activating other antioxidant mechanisms (MARUTA e colab., 2016). Indeed, recent works have reported that H_2O_2 generated in chloroplasts closely associated to nucleus are involved in the signalling for different antioxidative defense mechanisms and that this process is dependent on the inactivation of chlAPX isoforms (EXPOSITO-RODRIGUEZ e colab., 2017). The fact that these enzymes are not essential to photooxidative stress responses may be explained by the fact that they are promptly inactivated in the presence of H_2O_2 and should be completely dispensable or replaceable in detoxification mechanisms related to plant defence against excessive light. This idea is strengthened by the increased level of 2-Cys PRXs found in the *tapx sapx* double mutant under high light, possibly as an effective compensatory mechanism (KANGASJÄRVI e colab., 2008). However, whether other enzymes such as GPXs can also compensate the absence of chlAPXs remain to be determined.

GPX catalyzes the reduction of H_2O_2 and organic peroxides into water and the corresponding alcohols using reducing power provided by different reductants (Figure 1) (HERBETTE e colab., 2007; SELLES e colab., 2012). The GPX family is found in virtually all

kingdoms of life and has been increasingly studied in plants (MARGIS e colab., 2008; PASSAIA; MARGIS-PINHEIRO, 2015). This enzyme family is part of the heme-free thiolperoxidase class which can use glutathione, TRX and other reducing agents as substrates (HERBETTE e colab., 2002). The class-4 GPX, also named phospho-lipid GPX (commonly called PHGPX) can utilize organic hydroperoxide/H_2O_2 and GSH as oxidant and reductant, respectively, for its activity (MARGIS e colab., 2008; URSINI e colab., 1985). However, plant GPX isoforms exhibit higher affinity to use TRXs as electron donors (CHA e colab., 2008; HERBETTE e colab., 2002; WANG, Xin e colab., 2017) and the utilization of GSH as reducing agent by this enzyme has been questioned under *in vivo* conditions (HERBETTE e colab., 2002; IQBAL e colab., 2006; JUNG e colab., 2002; NAVROT e colab., 2006). Chloroplast GPX isoforms have been specially reported as important players for plant development and to cope against different environmental stresses in several species (KIM e colab., 2009; PASSAIA e colab., 2013; RODRIGUEZ MILLA e colab., 2003; WANG, Xin e colab., 2017), while non-chloroplast GPX isoforms play important role in photosynthesis (LIMA-MELO e colab., 2016). Additionally, single GPX proteins or the balance between reduced (GSH) and oxidized (GSSG) glutathione seems to act as redox sensors in plant cells (MIAO e colab., 2006; PASSAIA; MARGIS-PINHEIRO, 2015). As such the balance between GSH/GSSG and the activity of glutathione-related enzymes could regulate the chloroplastic redox metabolism.

d. The role of glutathione-related enzymes in the control of the redox metabolism

Given that glutathione is one of the major players in plant redox regulation, glutathione-related enzymes such as GPX, glutathione synthetase (GS), glutathione transferase (GST), and glutathione reductase (GR) are therefore important redox players in addition to the previously mentioned members of the plant antioxidant defense system. The role of glutathione-related enzymes has been well described regarding their roles in antioxidative mechanisms, especially under abiotic stresses (HARSHAVARDHAN e colab., 2017; SU e colab., 2016). These proteins are directly involved in the balance between reduced (GSH) and oxidized (GSSG) glutathione concentration, controlling thus the GSH redox state (BURRITT, 2017; FOYER, Christine H.; NOCTOR, 2013). More than that, some of these enzymes are also important for the redox metabolism because they can interplay directly with other proteins such as DHAR, GR, APX,

TRX, and PRX (BURRITT, 2017; NOCTOR e colab., 2012; PASSAIA; MARGIS-PINHEIRO, 2015).

Plant GSTs are spread in virtually all subcellular locations, including mitochondria, peroxisomes, nucleus, and mainly chloroplasts and cytosol (DIXON e colab., 2009; LALLEMENT e colab., 2014). GSTs catalyze the conjugation of GSH to electrophilic sites on a wide range of hydrophobic compounds (CHRONOPOULOU e colab., 2014; LABROU e colab., 2015). They can be sub-divided into 17 distinct classes (NIANIOU-OBEIDAT e colab., 2017) and can play a great variety of reactions, which include transferase, glutathionylation, peroxidase, isomerase, dehalogenation, and deglutathionylation activities (LALLEMENT e colab., 2014; SU e colab., 2016). The diversity of GSTs function is high and a special role of these proteins in the regulation of secondary metabolism has been postulated (DIXON; EDWARDS, 2010). In chloroplasts and cytosol, the most studied GST is dehydroascorbate reductase (DHAR), which is involved in the ascorbate regeneration from the Foyer-Halliwell-Asada cycle (ASADA, 1999; FOYER, Christine H; HALLIWELL, 1976).

The GR1 and GR2 enzymes belong to a NADPH-dependent oxidoreductase group of flavoproteins. GR1 is found in the cytosol whilst GR2 is found in both mitochondrion and chloroplast (CHEW e colab., 2003). These enzymes play an essential role in the maintenance of the GSH pool by catalyzing the reduction of one molar equivalent of GSSG to two molar equivalents of GSH. Taking into account that the balance of GSH/GSSG is an important indicator of the redox state of the cell, it was previously thought that GR activity could be of pivotal importance for controlling the redox potential of plant cells (DELORME-HINOUX e colab., 2016), especially in chloroplasts where the input of energy by light absorption leads to a highly dynamic and ROS-enriched environment (DING; JIANG e colab., 2016; WU e colab., 2015). However, plants lacking GR1 showed increased content of GSSG and this did not lead to higher stress sensitivity (MARTY e colab., 2009). This suggests that another system can compensate the absence of GR1. In fact, it has been shown that Arabidopsis NTRA/TRX *h3* system exhibit functional redundancy with cytosolic GR1 (MARTY e colab., 2009; REICHHELD, Jean-Philippe e colab., 2007). On the other hand, it was demonstrated that the function of GR1 in day length-dependent redox signalling cannot be replaced by the chloroplastic/mitochondrial isoform GR2 or by the TRX system (MHAMDI e colab., 2010). In contrast to *gr1* mutants, the knockout of GR2 is lethal in Arabidopsis (TZAFRIR e colab., 2004), whilst *gr2* knockdown plants presented early

leaf senescence, defective root development, and higher sensitivity to high light stress than WT, which was associates to PSII damage caused by ROS overaccumulation and by an altered GSH/GSSG redox status (DING; JIANG e colab., 2016; DING; WANG e colab., 2016; YU, X. e colab., 2013). It seems likely that GR1 acts in synchrony with the cytosolic NTR/TRX system while the effects of chloroplastic GR2 knockdown cannot be compensated by the chloroplastic NTR/TRX systems.

3 CROSSTALK BETWEEN CHLOROPLAST AND OTHER SUBCELLULAR COMPARTMENTS

In the previous section we have provided a brief overview regarding the function of the main players of the chloroplastic redox network. It is clear that the activity of the enzymes of this redox system is important to maintain the chloroplast metabolism in perfect harmony. However, it is important to highlight that the plant organelles work in concert, with several molecules being responsible for the communication between different subcellular compartments. Thus, the function of the different chloroplastic redox players is not limited to this organelle. In the following sections we highlight which chloroplastic molecules contribute to the inter-organellar communication and provide a perspective concerning how signals from and to chloroplast can coordinate the entire plant cell redox metabolism.

1.1. Chloroplast ROS-mediated signalling

Most redox components are highly active under light conditions. Light-dependent chloroplast reactions provide electrons via the PSI to ferredoxin which reduces either TRX via FTR or NADPH via FNR (MEYER e colab., 2009). The thiol reductase system is responsible for the regulation of a wide range of proteins and thus plays a pivotal role in the redox regulation of the plant cell. However, in parallel to the light-induced energy excitation, oxygen evolution in chloroplasts can lead to the formation of different ROS which are extremely harmful in high concentrations. Therefore, the ROS scavenging system which is mainly comprised of APX, GPX, SOD and PRX must work concurrently to the NTR/FTR-TRX system in order to maintain the

balance of ROS and thereby avoid overoxidation of the chloroplast (Figure 1). Alternatively, ROS can be transported to the cytosol where another antioxidant system takes place to eliminate the excess of these harmful molecules. Thus, the transport of ROS out of the chloroplast might act as a signal that connects the different compartments of the plant cell (MITTLER, 2017).

Among the different ROS, H_2O_2 has been credited as the most potent signaling molecule. This conception relies on some important characteristics presented by H_2O_2, including relatively low reactivity and higher half-life, as compared with other ROS (MØLLER e colab., 2007; POLLE, 2001). The recent discovery of a sub-population of chloroplasts closely associated with nuclei that are able to induce nuclear accumulation of H_2O_2 during the onset of light stress indicates a possible pathway for crosstalk and retrograde signaling mechanisms connecting photosynthesis and nuclear gene expression regulation (EXPOSITO-RODRIGUEZ e colab., 2017). This idea is strengthened by the fact that some yeast redox-sensitive thiol-disulphide transcription factors are activated by TRXs and peroxidases and deactivated by H_2O_2-mediated oxidation (FOMENKO e colab., 2011). It has been proposed that this redox-relay mediation mechanism should be also conserved in plants (EXPOSITO-RODRIGUEZ e colab., 2017). For instance, the redox-responsive transcription factors from the RAP2.4 family, which control the 2-Cys PRX and chlAPX expression in plants, presents cysteine residues that might be oxidized by H_2O_2 (RUDNIK e colab., 2017; SHAIKHALI e colab., 2008). This suggests that chloroplast signals are important to regulate nuclear gene expression which seems to be subjected to a H_2O_2-mediated redox regulation.

Beyond H_2O_2, it is noteworthy that other signals can also act as intermediates of interorganellar communication. For instance, the transport of malate from the chloroplast to the cytosol and subsequently to mitochondria has been proposed as a possible mechanism that connects these subcellular compartments (GEIGENBERGER e colab., 2017; HEYNO e colab., 2014). In the next section we discuss the role of malate metabolism in the interorganellar communication under different light/dark conditions.

a. The role of the circulating malate to both NAD(P)(H) metabolism and interorganellar communication under dark and light conditions

Malate metabolism regulates a wide range of physiological processes such as respiration, stomatal movements, fruit ripening, photorespiration, and seed maturation (ZHANG, Youjun;

FERNIE, 2018). Malate is found in different cell compartments and it seems likely that its function depends on the concentration and the location where it is found (FERNIE; MARTINOIA, 2009). For instance, it has been shown that malate act as a signalling molecule, respiratory substrate and a regulator of stomatal movements when located in cytosol, mitochondria, and apoplastic space, respectively (Araújo *et al.*, 2011; De Angeli *et al.*, 2013; Medeiros *et al.*, 2016, 2017). Thus, it is not surprising that malate metabolism is finely regulated among plant cell organelles. In this context, the chloroplastic malate valve has long been thought to have implications for the communication between chloroplast and other organelles. This idea is based on the fact that the chloroplastic NADP-dependent malate dehydrogenase (MDH) activity is strictly dependent on light and TRX activation and that malate is a circulating form of reducing power throughout plant cell (MICHELET e colab., 2013). It therefore seems likely that the transport and accumulation of malate in different subcellular compartments closely follow different environmental signals. Malate is thus a possible redox sensor that links different plant cell organelles according to the light/dark condition (Figure 3).

In the light, mitochondria respiration is inhibited (TCHERKEZ e colab., 2012) whilst both chloroplastic and cytosolic MDH are active. Thus, the amount of the circulating malate in the light is mainly due the activity of the non-mitochondrial MDH enzymes and the export of previously night stored organic acids from the vacuole (Figure 3). An active NADP-MDH is highly important to consume the excess of NADPH generated by the photosynthetic electron transport chain and to regenerate the electron acceptor $NADP^+$ (HARA e colab., 2006; HUANG e colab., 2018). Intriguingly, both cytosolic and chloroplastic MDH are clearly redox regulated by TRX (HARA e colab., 2006; THORMÄHLEN e colab., 2017; WOLOSIUK e colab., 1977; YOSHIDA e colab., 2015), while the mitochondrial MDH is irresponsive to TRX (DALOSO e colab., 2015b; HUANG e colab., 2018; YOSHIDA; HISABORI, 2016a). Perhaps more challenging is the fact that fumarase (FUM) is activated by TRX *h2* and deactivated by TRX *o1 in vitro* (DALOSO e colab., 2015b), suggesting that FUM can be positively and negatively regulated by different TRXs (Figure 3). However, given the absence of data from *trxh2* mutants and the dual location of TRX *h2*, which is found in both cytosol and mitochondria, it is still unclear whether the TRX *h2*-mediated FUM regulation also occurs *in vivo* and if so in which subcellular compartment this occurs. Another outstanding question is why would FUM and cytosolic MDH be simultaneously activated by TRX *h2*. Could the TRX-mediated activation of

both FUM and cMDH act as a mechanism for the synthesis of fumarate in the cytosol? This idea is supported by the fact that the carbon fluxes to the TCA cycle are light-inhibited, which thus compromise the synthesis of organic acids in mitochondria, and also by the fact that the massive accumulation of fumarate in the light depends on the cytosolic FUM (FUM2) activity (PRACHAROENWATTANA e colab., 2010), which is higher in the direction of fumarate synthesis and activated by Gln, Asn and OAA (Figure 3) (ZUBIMENDI e colab., 2018). The questions raised here can be solved by the analysis of mutants lacking different TRX isoforms (discussed latter in the section 5) and by non-aqueous fractionation metabolic analysis that can determine the accumulation of malate and fumarate in different subcellular compartments (KRUEGER e colab., 2014; MEDEIROS e colab., 2017). Thus, further experiments in this direction must assume a paramount importance to elucidate the role of malate and malate valve for the cross-talk between chloroplasts and mitochondria in leaves exposed to light.

By contrast to the accumulated knowledge regarding light/dark reactions in the chloroplasts, information concerning light/dark regulation of mitochondrial enzymes remains very scarce (NIETZEL e colab., 2017). It is reasonable to assume that chloroplastic malate valve may only have a minor impact on the redox regulation of plant cells in the dark given that the input of energy through photosynthesis is absent and NADP-MDH is inactive (Figure 3). However, another redox mechanism mediated by NTRs (NTRA and NTRB) and TRXs (TRX *h* family) can activate cytosolic MDH in the cytosol (HARA e colab., 2006; HUANG e colab., 2018) In this case the source of NADPH for NTRs comes from the oxidative pentose phosphate pathway (OPPP). Mitochondria also contain both NTRA and NTRB enzymes and different TRXs (TRX *o1*, *o2*, *h2*). In this organelle, NADPH can be supplied by the activity of a NADP-dependent isocitrate dehydrogenase (ICDH) and alternatively by a presumable mitochondrial NAD(H) kinase (NADk) (Figure 3) (GEIGENBERGER e colab., 2017; MØLLER; RASMUSSON, 1998; MØLLER, 2001; RASMUSSON e colab., 2004). Interestingly, ICDH-dependent NADPH production seems to be redox regulated by TRX (YOSHIDA; HISABORI, 2014). Beyond ICDH, other TCA cycle-related enzymes such as citrate synthase (CS), FUM, and succinate dehydrogenase (SDH) as well as the alternative oxidase (AOX) have already been suggested to be TRX-regulated (DALOSO e colab., 2015b; GELHAYE; ROUHIER; GERARD e colab., 2004; SCHMIDTMANN e colab., 2014; YOSHIDA; HISABORI, 2014). Recent evidence indicated that TRX *o1* controls the flux of C to the TCA cycle in the light by deactivating both

FUM and SDH (DALOSO e colab., 2015b). This result, together with the light inhibition of pyruvate dehydrogenase (PDH) (TOVAR-MÉNDEZ e colab., 2003) and the role of mitochondrial MDH, which seems to act preferentially in the synthesis of malate to be exported to cytosol (TOMAZ e colab., 2010) (Figure 3), could explain why respiration is inhibited in the light. Furthermore, the light inhibition of FUM1, SDH and PDH also helps to explain previous predictions from a genome scale metabolic model (CHEUNG e colab., 2014) and recent results from a nuclear magnetic resonance-based metabolic flux study that indicate a non-cyclic mode of operation of the TCA cycle in the light (ABADIE e colab., 2017). The results from these studies suggest that the TCA cycle works in a non-cyclic mode to sustain glutamate (Glu) and glutamine (Gln) biosynthesis in the light, in which TRXs would be key regulators by activating CS and ICDH and deactivating FUM and SDH (Figure 3). In turn, Gln biosynthesis would activates FUM2 leading to the synthesis of fumarate in the cytosol. However, whether these reactions also occur in the dark remain to be seen.

In summary, it seems likely that both NAD(P)(H) and malate metabolism, especially the malate valve and the activities of MDH and FUM, are important players for the crosstalk between chloroplasts and other organelles. It has been shown that alteration of key chloroplastic, mitochondrial, or peroxissomal enzymes alter not only the processes that take place autonomously within each organelle but also have high impact in processes occurring in other organelles. This suggests that the different plant cell organelles are tightly regulated. In the next section we explore the mechanisms and players for this connection between plant subcellular compartments.

b. Chloroplastic and mitochondrial metabolism are tightly regulated

The characterization of plants lacking or displaying reduced activity of key enzymes of the redox metabolism has demonstrated that the perturbation of some chloroplastic enzymes affects different processes in other subcellular locations. Similarly, perturbation in mitochondrial enzymes has also been demonstrated to alter chloroplastic metabolism. The cross-regulation between chloroplast and mitochondria is probably the most studied interorganellar system in plants (BLANCO e colab., 2014; MIGNOLET-SPRUYT e colab., 2016; NOCTOR e colab., 2018; UHMEYER e colab., 2017). Special attention has been given to the mitochondrial enzyme AOX, given its importance for both mitochondrial respiration and in maintaining chloroplast

redox homeostasis and photosynthetic rates (DAHAL; VANLERBERGHE, 2017; DEL-SAZ e colab., 2018b; FLOREZ-SARASA; NOGUCHI e colab., 2016; VISHWAKARMA e colab., 2014; WELCHEN; GONZALEZ, 2016). Several other mitochondrial proteins involved with the TCA cycle and mitochondrial electron transport chain have additionally been shown to regulate chloroplast metabolism (PIRES e colab., 2016; SWEETLOVE e colab., 2006; TIMM, Stefan e colab., 2012a, 2018). However, little is known about the importance of enzymes of the redox network such as APXs, GPXs, and TRXs among others in this interorganellar regulation. Recent evidence showed that the silencing of a mitochondrial glutathione peroxidase (OsGPX1) penalizes photosynthetic assimilation and growth rates in rice by a mechanism involving changes in cell H_2O_2 and GSH contents (LIMA-MELO e colab., 2016). Similarly, plants lacking the mitochondrial TRX *o1* presented high activities of enzymes of the redox metabolism such as SOD and catalase under salt stress (CALDERÓN e colab., 2018) beyond up regulation of AOX (DEL-SAZ e colab., 2016). Taken together, these studies suggest the perturbation of mitochondrially located GPX or TRX had consequences for plant cell redox metabolism in general. On the other hand, mutants lacking specific chloroplast proteins have been used to investigate the consequences on mitochondrial metabolism. For example, it was demonstrated that Arabidopsis mutants lacking proteins related to the cyclic electron flow display higher AOX activity and changes in NAD(P)/NAD(P)H ratios under high light (FLOREZ-SARASA; NOGUCHI e colab., 2016). Therefore, it seems that both chloroplast and mitochondrial metabolism are tightly regulated, by a mechanism which may involve the transmission of signals such as ROS, nitric oxide and calcium as well as the accumulation of shared metabolites such as malate and fumarate (ARAÚJO; NUNES-NESI; FERNIE, 2011; DEL-SAZ e colab., 2018a; FERNIE; MARTINOIA, 2009; FLOREZ-SARASA; RIBAS-CARBO e colab., 2016). Further studies will likely prove important in unraveling the connectors between these organelles as well as the importance of this communication for plant stress tolerance.

c. Cytosol as a redox buffer and an interorganellar communication mediator

Experimental evidence has accumulated that the plant cytosol might represent a crucial cellular compartment for the metabolic regulation of the whole cell, acting as a redox buffer and an important crossroad for retrograde signaling pathways. Indeed, the cytosolic APX and ASC-GSH cycle display a central role in the scavenging of excessive H_2O_2 produced by other

organelles such as peroxisomes, chloroplasts and mitochondria (Davletova *et al*., 2005). The cytosol contains the majority of the antioxidant defense protein isoforms, comprising a dynamic system involved in ROS production and scavenging in a manner that might affect both local and systemic responses (MUNNE-BOSCH e colab., 2013). Furthermore, cytosol also contains a complete NTR/TRX system composed by NTRA/NTRB and different TRX *h* proteins. The buffering capacity of cytosol is evidenced by the fact that the excess of cytosolic H_2O_2 generated by APX deficiency in Arabidopsis inhibited photosynthesis by inducing oxidative stress in chloroplasts (DAVLETOVA, 2005). The excess ROS can cause denaturation of crucial Calvin-Benson cycle proteins by carbonylation, including Rubisco (Davletova *et al*., 2005), and even delays in the PSII repair process (MURATA; NISHIYAMA, 2018). Together, these damages can induce a strong restriction of CO_2 assimilation and photoinhibition of PSII, contributing to a general impairment in photosynthetic capacity and plant growth (FOYER, Christine H. e colab., 2012). Recently, we have demonstrated that accumulation of peroxisomal H_2O_2 induced by CAT inhibition and APX knockdown in rice plants also deeply affected the cytosolic antioxidant protein synthesis, especially in those enzymes involved in ASC-GSH cycle (unpublished data). Besides cytosolic H_2O_2, other signaling molecules produced by several different metabolic pathways such as GSH might trigger retrograde signaling, which in turn may alter several redox responses in the distinct plant cell compartments (KÖNIG e colab., 2018). In this vein, the photorespiratory pathway consists in an important route for the generation of exchangeable signaling molecules, especially for connecting chloroplasts, cytosol, peroxisomes and mitochondria (TIMM, S. e colab., 2013).

d. Beyond a wasteful pathway: the important role of the photorespiratory metabolism to photosynthesis and for the maintenance of plant cell redox state

In addition to chloroplasts and mitochondria, the peroxisome is also an important organelle involved in ROS-related signaling (DEL RÍO e colab., 2003; DIETZ, Karl Josef, 2015; NOCTOR; FOYER, 2016). Chloroplasts and peroxisomes are connected by the process of photorespiration, in which the oxygenase activity of the chloroplastic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) induces the photorespiratory pathway that also involves mitochondria (BAUWE e colab., 2010). Furthermore, peroxisomes have been described as the main source of ROS in plant cells, especially in C3 leaves exposed to light (FOYER,

Christine H.; NOCTOR, 2003). A study with Arabidopsis mutant plants overexpressing glycolate oxidase in the chloroplast and plants deficient in peroxisomal catalase showed that the H_2O_2-dependent signal is different when this ROS is generated in chloroplast from that generated in peroxisomes (SEWELAM e colab., 2014). Additionally, it was shown that plant cells have an integrated signal network that works independently of the subcellular site of H_2O_2 production (SEWELAM e colab., 2014). These reports show the importance of understanding redox and ROS signaling pathways originated from different subcellular compartments, especially those from peroxisomes. In this context, RNAi suppression of both peroxisomal rice APX isoforms coupled with a pharmacological inhibition of catalase triggered favorable antioxidant and compensatory mechanisms to cope with oxidative burst conditions, most probably due to a priming mechanism induced by peroxisomal H_2O_2 signalling (SOUSA e colab., 2015). These results suggest that peroxisomal H_2O_2-mediated signalling has a pivotal role for the maintenance of redox state of the whole plant cell.

Photorespiration has long been described as a waste pathway that competes with the carboxylase activity of RubisCO and thus reduces the yield in C3 plants. With this idea in mind, several research groups have engineered plants to improve plant yield by suppressing photorespiration or by increasing the carboxylation-to-oxygenation ratio of RubisCO. However, recent reports showed that the overexpression of some photorespiratory enzymes leads to increased photosynthesis and plant growth (TIMM, Stefan e colab., 2012b, 2015) and that photorespiration is crucial for the photosynthetic and stomatal responses to CO_2 availability (EISENHUT e colab., 2017). These findings indicate that the photorespiratory metabolism is important for the control of photosynthesis. Photorespiration may contribute to increased photosynthetic rate by eliminating toxic intermediates, recycling substrates for RubisCO carboxylation, and providing substrates for other metabolic pathways (WINGLER e colab., 2000). Moreover, it has been shown that the activity of Calvin-Benson cycle enzymes are regulated by the accumulation of the photorespiratory metabolite 2-phosphoglycolate (FLÜGEL e colab., 2017). Thus, given that photorespiration encompasses transport of metabolites between chloroplasts, peroxisomes, cytosol and mitochondria and involves the production of H_2O_2 in peroxisomes and NADH in mitochondria, it is reasonable to attribute a great importance to this pathway in the regulation of the entire plant cell redox network (GEIGENBERGER; FERNIE, 2014a; OBATA e colab., 2016; TIMM, Stefan e colab., 2016). It is therefore clear that the

function of each enzyme of the plant cell redox system is not specific to the organelle where that reaction takes place, suggesting that the entire redox system acts in synchrony. Evidence for this comes from studies that have revealed several compensatory and redundant roles among the redox players, which has been mainly discovered by the characterization of plants lacking multiple isoforms of a redox system. In the next section, we review these findings and provide a perspective on which hypothesis should be tested in the near future to improve our knowledge concerning how the different redox components interact and compensate to each other.

4 REVERSE GENETIC STRATEGIES TO UNRAVEL REDUNDANT AND COMPENSATORY MECHANISMS BETWEEN REDOX NETWORK COMPONENTS

The redundancy and the compensatory role observed among the players of the redox metabolism is mainly perceptible by studies that use specific inhibitors coupled with reverse genetic approaches (Rizhsky *et al.* 2002; Sousa *et al.* 2015; Bonifacio *et al.* 2016; Rahantaniaina *et al*., 2017)(SOUSA e colab., 2015)(SOUSA e colab., 2015) or by the characterization of mutants that lack the activity of more than one protein of the redox network (BASHANDY e colab., 2009; DALOSO e colab., 2015b; MARTY e colab., 2009; PÉREZ-RUIZ e colab., 2017; REICHHELD, Jean-Philippe e colab., 2007; THORMÄHLEN e colab., 2015; YOSHIDA; HISABORI, 2016b). Recent reports have used these strategies and were able to provide considerable information as to how the different components of the chloroplastic redox network can compensate for one another. For instance, it is known that the reduced growth rate of both the *ntrc* mutant and the *ntrc trxf1 trxf2* triple mutant is due the overaccumulation of oxidized 2-Cys PRX which supposedly acts as a sink for reducing power from the chloroplastic TRXs (PÉREZ-RUIZ e colab., 2017). Furthermore, despite the essentiality of NTR for mammalian systems (CONRAD e colab., 2004), the *ntrc* mutant and the *ntra ntrb* double mutant are still viable, most probably due a compensatory role of FTR system in chloroplasts and GR and glutathione metabolism in cytosol and mitochondria (BASHANDY e colab., 2009; DALOSO e colab., 2015b; MARTY e

colab., 2009; REICHHELD, Jean-Philippe e colab., 2007; YOSHIDA; HISABORI, 2016b). It seems clear therefore that different redox components of each organelle could act in concert to maintain its redox state in perfect harmony. However, how the redox systems from different compartments interact with one another remains unclear. For instance, it remains to be investigated as to whether plants are still viable with the knockout of the entire plant NTR system (Table 2).

Despite recent advances, relatively few studies have simultaneously disrupted chloroplastic and non-chloroplastic redox components, a fact which hampers our understanding on how these systems compensate to each other. We propose here that the establishment and characterization of a number of mutants lacking different components of the plant cell redox network will certainly provide great information for the plant redox scientific community. Given that the compensation of components of the chloroplastic redox network might be achieved by non-chloroplastic redox components, different combinations of mutations should be carried out (Table 2). For instance, assuming that TRX *o2* is mitochondrially located, the characterization of the *trxo1 trxo2 trxh2* triple mutant, which will supposedly present no mitochondrial TRX activity, may reveal the possible redundancy between these TRXs and enable the investigation whether other enzymes such as mitochondrial GRXs can compensate the absence of these TRXs. Furthermore, given the already described role of TRX *h* proteins in the activation of cMDH and FUM2, it is reasonable to assume that the characterization of plants lacking multiple TRX *h* proteins may provide important information regarding how these TRXs regulate malate metabolism and whether GRXs can compensate the absence of these proteins (Table 2).

In the case of chloroplastic peroxidases, few studies have investigated double or triple mutants. Thus, different combinations to knockout sAPX, tAPX, and GPXs would be anticipated to provide important insights. All other possible combinations which include plants lacking or deficient in more than one enzyme of the chloroplastic reductase system (NTRC, FTRA, FTRB, and GR2) are described in detail in the Table 2. After the establishment of plants lacking different enzymes of the redox network, it is important to highlight that the processes regulated by these enzymes are better understandable by adopting systems biology approaches. In the next section we provide a perspective with two practical examples on how systems biology approaches can be used to improve our understanding on the function and interactions among redox-related enzymes.

5 USING SYSTEMS BIOLOGY APPROACHES TO UNRAVEL INTERACTION AND CROSS- REGULATION AMONG ENZYMES OF THE CHLOROPLASTIC REDOX NETWORK

The emergence of omic platforms in the last decades has produced vast quantities of data which are currently available for bioinformatics and modelling analysis. Plant biology thus became a data-enriched, multidisciplinary field in which the application of mathematics, physics, and computational biology concepts are proving to be essential for integrating and understanding the experimental data acquired. This multidisciplinary scientific field is based on the ideas from the general systems theory (VON BERTALANFFY, 1968) and it is commonly referred to as systems biology (FRIBOULET; THOMAS, 2005). The aim of systems biology is to understand the dynamic of the interactions between the different networks that compose a complex organism such as plants (BARABÁSI; OLTVAI, 2004; SHETH; THAKER, 2014). For this, different systemic approaches can be adopted in order to provide a holistic view of plant responses by contrast of the reductionism approaches that are mainly focused in looking at the parts (FERNIE, 2012; SOUZA e colab., 2016). Systems biology approaches have been successfully applied to the study of gene expression and metabolic networks in plants (TOUBIANA e colab., 2013), which resulted in several mathematical models able to predict plant metabolic responses (HILLS e colab., 2012; NIKOLOSKI e colab., 2015; ROBAINA-ESTÉVEZ e colab., 2017; WILLIAMS e colab., 2010). However, the application of such systems biology approaches has been surprisingly neglected in the study of redox metabolism. In this section we carried out different gene co-expression (Figure 4) and protein-protein interaction network (Figure 5) analysis and discuss the main findings from these analyses as well as how these approaches can help to understand the interaction between the components of the chloroplastic redox metabolism. Although these approaches have been widely used in plant biology, it is important to highlight the limitation of gene expression and specially protein-protein interaction databases, which are mostly limited to model plants, in particular Arabidopsis, beyond the intrinsic limitation of *in silico* analysis that needs further experimental validation. In this context, Arabidopsis protein-protein interaction databases are still fragmentary and thus caution should be taken when evaluating the results coming out from this analysis. Among the problems faced by this analysis

it is noteworthy the presence of false positives interactions as well as the absence of protein-protein interactions not detected by the Arabidopsis interactome study.

1.1. Gene co-expression analysis reveals a highly connected network among the components of the chloroplastic redox system

Gene co-expression and protein-protein interaction network analyses were carried out using all chloroplastic isoforms of TRXs, PRXs, GRXs, GR, NTR, FTRs, APXs, and GPXs, which is hereafter referred to as chloroplastic redox network. Interestingly, the gene co-expression analysis revealed that these genes are highly co-expressed, leading to a highly dense, inter-connected network (Figure 4). Surprisingly, both tAPX and sAPX are not co-expressed to each other and slightly co-expressed with other genes of the chloroplastic redox network. For instance, tAPX is only co-expressed with PRXQ, NTRC, GR2, and both 2-Cys PRX. Moreover, the Arabidopsis interactome database (ARABIDOPSIS INTERACTOME MAPPING CONSORTIUM, 2011) does not show any interaction for sAPX isoform, suggesting that whether sAPX isoform is regulated by mechanisms linked to the chloroplastic redox network this occurs in an indirect manner.

1.2. Is plant gene expression redox-regulated?

(NAVROT e colab., 2006; PASSAIA; MARGIS-PINHEIRO, 2015; PELTIER e colab., 2006)(NAVROT e colab., 2006; PASSAIA e MARGIS-PINHEIRO, 2015; PELTIER e colab., 2006) *In silico* analyses revealed that GRX and GPX are highly connected nodes in the co-expression network. At the protein level, GRXS14 seems to be a hub-like node, i.e. a node (enzymes) with high number of links (interaction) (BARABÁSI; OLTVAI, 2004). GRXS14 directly interacts with five proteins, including plastidial transcription factor 1 (PTF1), a chloroplastic transcription factor (TF) (BABA e colab., 2001), which was linked to different other important proteins of the chloroplastic redox network such as NTRC and TRX *y1* (Figure 6). This suggests that PTF1 is potentially redox regulated by different components of the chloroplastic redox network. This has considerable consequences for chloroplast function given the extremely high number of protein-protein interactions detected for PTF1 (Figure 6). It has been shown that TRXs can directly activate or deactivate redox-sensitive TFs in mammalian cells (POWIS; MONTFORT, 2001; SCHENK e colab., 1994; SUN; OBERLEY, 1996). However, this post-translational control of TF activity

remains relatively less studied in plants (FARNESE e colab., 2016). It is known that HD-Zip proteins and Class I TCP TF are oxidized by H_2O_2, GSSG and other oxidants and reduced by the NTR/TRX system (COMELLI; GONZALEZ, 2007; VIOLA e colab., 2013). Similarly, several TFs are suggested to be redox-regulated (SHAIKHALI; WINGSLE, 2017). Thus, it seems that plants resemble animals in the redox regulation of transcription through a TRX-mediated (de)activation of TFs. Our *in silico* analysis provides new insights into transcription regulation in plants by suggesting TFs which interact with NTRC, TRXs, and GRXs and thus might be subjected to a redox regulation. Efforts to confirm this hypothesis assume a paramount importance for future investigation.

1.3. TRX h3 is a putative regulator of different cytosolic proteins

Proteins from the TRX *h* family (TRX *h1-9*) have been extensively studied in seeds (HÄGGLUND e colab., 2016; SHAHPIRI e colab., 2009). It has been proposed that the majority of TRX *h* proteins are located in the cytosol (BRÉHÉLIN e colab., 2004; GEIGENBERGER e colab., 2017), although TRX *h2* and TRX *h9* has been demonstrated to be located at the mitochondria and associated to the membrane, respectively (MENG, Ling e colab., 2010). TRX *h3* is the highest expressed among TRX *h* genes (REICHHELD, Jean Philippe e colab., 2002) and has long been recognized as a cytosolic isoform (GELHAYE; ROUHIER; JACQUOT, 2004; ITO e colab., 2011; PARK e colab., 2009), although this protein has also been identified in two different chloroplast proteomic studies (PELTIER e colab., 2006; ZYBAILOV e colab., 2008).

TRX *h3* is the highest expressed among TRX *h* genes (REICHHELD, Jean Philippe e colab., 2002). The Arabidopsis interactome revealed that TRX *h3* interacts with 50 proteins that can be clustered in seven different groups according to their function in plant cells. Intriguingly, five of these groups are chloroplastic enzymes related to photosynthesis, C and N metabolism, redox enzymes, and carbonic anhydrases (Figure 7). TRX *h3* supposedly interacts with the chloroplastic redox-related enzymes GST F8, DHAR3, Prx IIE, MSR A4, Fdx 1 and Fdx 2 (Figure 7). Furthermore, different photosynthetic proteins related to the CBC (e.g. SBPase, TK, R5Pepi, PRK, FBAldolase, RubisCO activase, CP12) and the oxygen evolution complex (OEC) seem to interact with TRX *h3* (Figure 7). However, it is important to highlight that there is no evidence confirming that TRX *h3* is a chloroplastic-located TRX. By contrast, TRX *h3*-GFP assay indicates that this protein is in fact located in the cytosol (PARK e colab., 2009). Thus,

despite the high number of interactions with chloroplastic proteins, we argue that this might be false-positive results or that these proteins interact with TRX *h3* during their translocation to the chloroplast. The other two groups that interacted with TRX *h3* in our analysis include cytosolic enzymes such as APX1, MDH, GAPDH and FBPase and nine other proteins with unknown function or unknown subcellular location. Interestingly, cytosolic MDH, ICDH, FBPase and GAPDH all interacted with TRX *h3*. This suggests that TRX *h3* may serve as a key regulator of C metabolism in the cytosol.

The Arabidopsis interactome also showed that TRX *h3* interacts with three β carbonic anhydrases (βCA1, βCA2, and βCA4). These enzymes are key regulators of stomatal movements in response to CO_2 (ENGINEER e colab., 2016). βCA1 and βCA4 are highly expressed in guard cells and the *ca1 ca4* double mutant has impaired stomatal responses to CO_2 (HU e colab., 2015). Redox regulation of carbonic anhydrases has already been demonstrated in the marine diatom *Phaeodactylum tricornutum* (KIKUTANI e colab., 2012). These facts suggest that βCAs and consequently stomatal responses to CO_2 can be redox regulated by TRX *h3*. However, confirmation that TRX *h3* regulates these enzymes *in vivo* remains to be experimentally assessed. The characterization of plants lacking TRX *h3* may bring important information concerning the general function of this protein in the regulation of stomatal movement and C metabolism (Table 2).

1.4. Lethality and centrality in chloroplastic redox network

Systemic analyses are important to identify essential nodes of biological networks, in which its removal from the network leads to disturbed or abolished physiological responses which can lead to the death of the organism (JEONG, Hawoong e colab., 2001; LI e colab., 2006). Hubs have been described as essential nodes of protein-protein interaction networks, in which the mutation in genes coding these proteins are lethal or have severe consequences for the organism (ALBERT, R, 2005; JEONG, Hawoong e colab., 2001; YU, H. e colab., 2008). By contrast, depletion of lightly connected nodes in scale-free networks has minor impact throughout the network and in consequence to the organism (BARABÁSI; OLTVAI, 2004). In this context, the most connected nodes of the co-expression network are NTRC and PRXQ with 27 links each followed by GR2 and the two 2-Cys PRX A and B with 25 links each (Figure 4). In the case of protein-protein chloroplastic interaction network, NTRC and TRX *y1* was shown to be the main

hubs, presenting 10 and 12 interactions, respectively, including the interaction between themselves (Figure 5)(NIKKANEN e colab., 2016)(NIKKANEN e colab., 2016). According to the centrality and lethality theory of scale-free networks (BARABÁSI; OLTVAI, 2004; JEONG, Hawoong e colab., 2001), hubs are very important components that confer robustness to biological networks (ALBERT, R, 2005; ALBERT, Réka e colab., 2001). Therefore, the expectation is that mutation in hubs may substantially alter the topology of the network which may have severe consequences for the organism. Indeed, plants lacking NTRC or GR2 demonstrate a drastic reduction in growth and/or high susceptibility to stress conditions (DING; WANG e colab., 2016; THORMÄHLEN e colab., 2015), most probably due to the perturbation of the chloroplastic redox network caused by those mutations. However, plants lacking PRX Q and TRX *y1* did not show any distinguishable phenotype compared to the WT (PETERSSON e colab., 2006). In the case of *ntrc* mutant, reduced growth in comparison to WT was observed, probably due to the overaccumulation of oxidized forms of 2-Cys PRXs (PÉREZ-RUIZ e colab., 2017), highlighting the interconnection of these genes that are co-expressed (Figure 4) and also interact to each other at protein level (Figure 5).

Taken together, these observations suggest that these hub-like proteins may have central roles in the chloroplastic redox network. In fact, NTRC is crucial for the activation of enzymes of CBC, antioxidative defence system, synthesis of starch, ATP and chlorophyll. Furthermore, NTRC are known to regulate chloroplast gene expression. Although less studied, TRX *y1* has been also implicated in the activation of the antioxidative defence system and chlorophyll and starch synthesis and degradation (GEIGENBERGER e colab., 2017). However, despite the centrality of NTRC in the chloroplastic redox network, by contrast to animal cells (CONRAD e colab., 2004), single mutation in any NTR protein is not lethal in plants. This suggests that the lethality and centrality theory of scale-free networks may have a further level of complexity in plant networks. Probably, the higher phenotypic plasticity of plants, which have been accomplished by, for example, an increased number of gene duplications, ultimately leads to the formation of different compensatory mechanisms that overcome the mutation in central nodes and avoid the propagation of the negative effects of the mutations throughout the whole network. This idea is based on the fact that double mutation in both components of the chloroplastic TRX reductase systems, named NTRC and FTR, is lethal in Arabidopsis (Yoshida and Hisabori, 2016), indicating that the absence of lethality in the *ntrc* mutant is probably due to a

compensatory role performed by the FTR. Similar to this phenomenon, several other components of the redox network can compensate to each other and thus explain the absence of apparent phenotype, justifying the need to characterize plants lacking different components of the redox system simultaneously. Further experiments are needed to confirm that the theory of lethality and centrality differs in plant from animal networks as well as to test the hypothesis that the higher phenotypic plasticity of plants are related to a lesser degree of lethality in the hubs of their networks.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant redox network possess a higher degree of complexity when compared to animal and microorganismal redox networks. This is evidenced by the higher number of isoforms of each component of the redox network and the complementarity observed between them. Whilst mutation in key components of the redox system is lethal or, more commonly, has severe consequences for animal cells, several single Arabidopsis or rice mutants do not show any distinguishable phenotype than non-transformed plants. In evolutionary terms, this may be an adaptive feature acquired by plants to grow and survive under constant adverse conditions which is a common situation of plant life due to their sessile nature. The higher number of isoforms of redox components may be therefore a mechanism that cooperatively adjust plant cell metabolism to avoid oxidative stress under a constant input of energy through the process of photosynthesis. Thus, both the redox regulation of metabolism and the regulation of the redox metabolic network itself have been singled out as important mechanisms for plant growth and plant stress tolerance. However, despite the fact that the understanding of the redox regulation of metabolism under stress conditions has received great attention, little is known concerning how the components of redox metabolism are regulated and interact with each other. We contend that this is mainly based on our failure in consider this regulation from a systemic perspective. As such adopting systems biology approaches may help to fill some of these gaps. In parallel, the redundancy and the compensatory role among the components of the redox network may eventually be completely unravelled by using multi-transgene approaches. Such strategies will likely assume a paramount importance in improving plant metabolic engineering for stress tolerance.

Acknowledgments

Scholarship granted by the Coordination for the Improvement of Higher Level Personnel (CAPES, Brazil) to P.V.L.S. and fellowship granted by the National Council for Scientific and Technological Development (CNPq, Brazil) to D.M.D. are gratefully acknowledged.

Figure 1 - Schematic representation of the chloroplastic redox network. This network is composed by different components mainly related to reduce/oxidize proteins and to remove the excess of stress-generated reactive oxygen species (ROS). Stress conditions such as high light, drought, and oxidative stress between others can leads to an overproduction of the harmful ROS singlet oxygen ($2O_2^-$) and hydrogen peroxide (H_2O_2). Superoxide Dismutase (SOD) is responsible to convert2 O_2^- in H_2O_2 which can be metabolized by different redox systems including ascorbate and glutathione metabolism and peroxiredoxins (Prx). For instance, glutathione reductase (GR) uses chloroplastic electron transport chain (ETC)-derived NADPH to convert oxidized glutathione (GSSG) in reduced glutathione (GSH). After that, GSH reduces glutathione peroxidase (GPX) which then converts H_2O_2 in H_2O. Similarly, ascorbate peroxidase (APX) is also capable to convert H_2O_2 in H_2O but using reducing power from the ascorbate cycle that includes the interconversion of ascorbate and monodehydroascorbate (MDHA) with help of the enzyme monodehydroascorbate reductase (MDHAR). Another pathway that leads to H_2O_2 degradation is by the activity of Prx that consist a family of antioxidant proteins. The degradation of H_2O_2 by 2-Cys Prxs seems to involve different state of oxidation/reduction of these proteins given that they can be oxidized (2-Cys Prxox) or overxidized (2-Cys Prxoox) by H_2O_2 and rescue their reduction state by the activity of sulfiredoxin (Srx) and/or NADPH-dependent thioredoxin reductase C (NTRC) and thioredoxins (TRXs). In parallel, the chloroplastic electron transport chain (ETC)-generated NADPH can be used to sustain the NTRC-TRX system that are able to reduce different proteins of the redox network (chech the S-S disulfide bound in the enzymes already showed to be TRX-mediated redox regulated).The chloroplast ETC is activated by light and leads to the reduction of ferredoxin (Fd) via ferredoxin NADPH+ reductase (FNR). Reduced Fd is then used by ferredoxin thioredoxin reductase (FTR) that together with NTRC can then reduce TRXs. GPX, malate dehydrogenase (MDH) and methionine sulfoxide reductase (MSR), whose function is to reduce methionine sulfoxide (MetSo) into methionine (Met), are examples of TRX-mediated redox regulated enzymes.

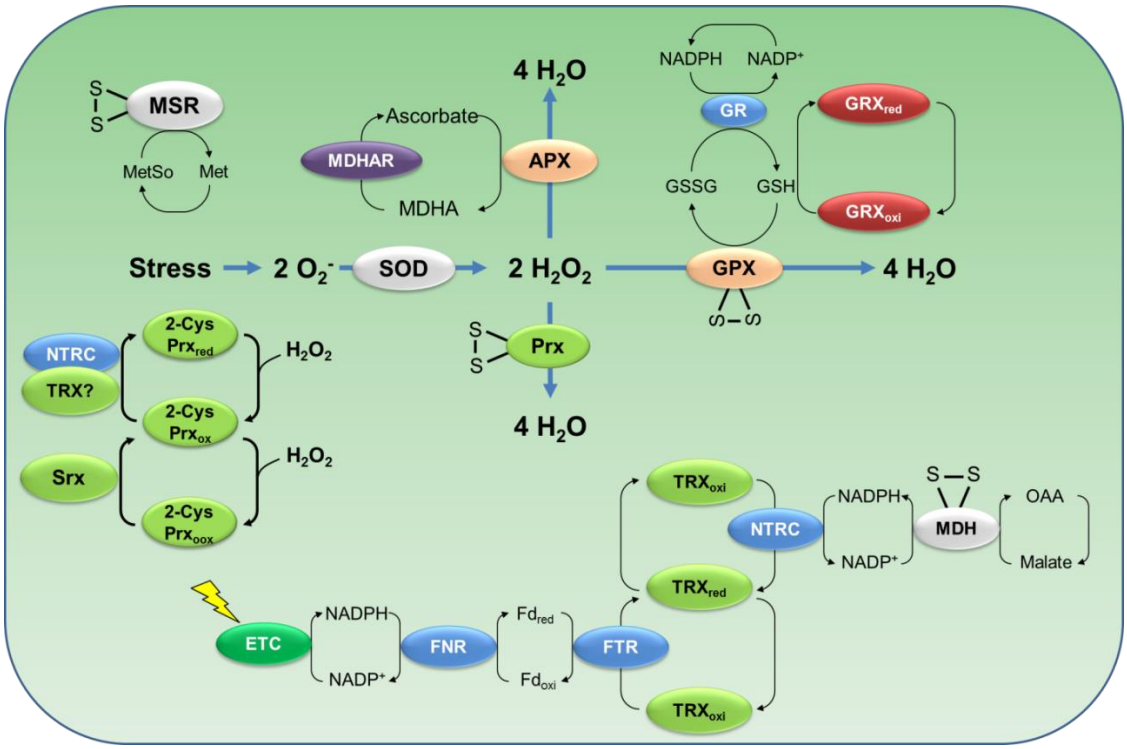

Figure 2 - Plant thioredoxin (TRX)-mediated redox regulation cascade. The TRX system is composed by reductases, TRXs and their target proteins (right panel). The left panel demonstrate the relative number of proteins of each of these components of the TRX system. The reductases NADPH-dependent TRX reductase (NTR), ferredoxin reductase (FTR), and glutathione reductase (GR) are responsible to reduce the different isoforms of TRXs, which are divided in TRXs f, h, m, o, x, y, and z. Reduced TRXs can then (de)activate a wide range of target proteins.

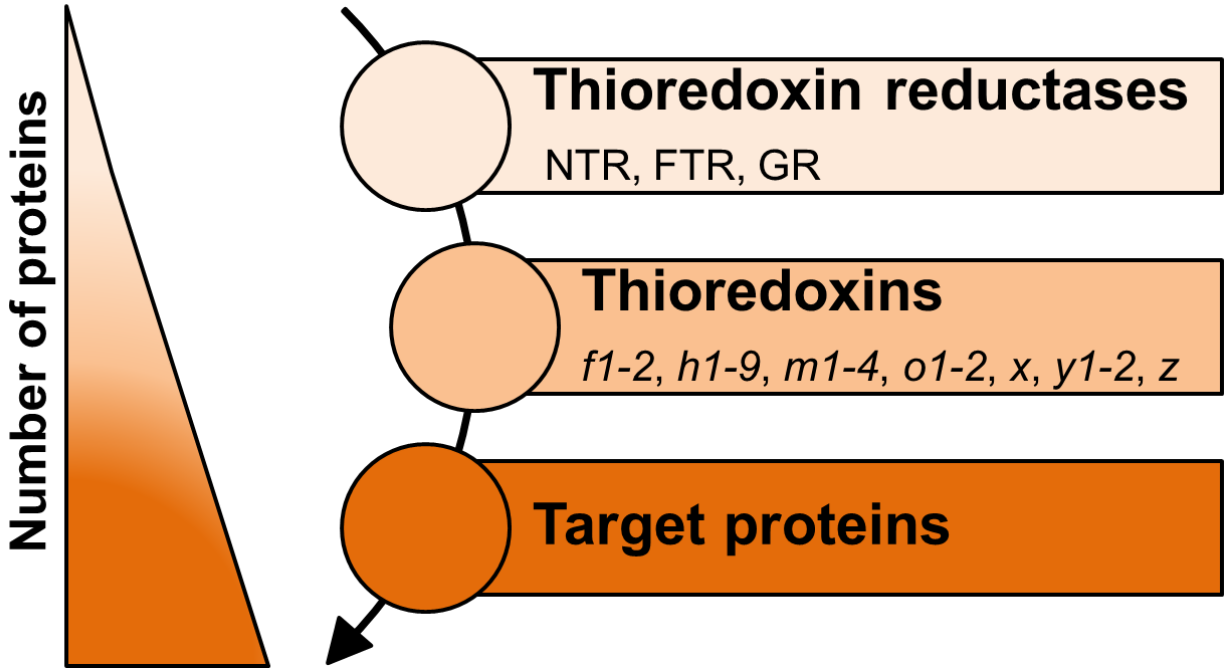

Figure 3 - Co-expression network between the main components of the chloroplastic redox protein network. The nodes (enzymes) are connected to each other when their genes are co-expressed. Gene co-expression network was carried out using String database platform (SZKLARCZYK e colab., 2017). The co-expression gene network was generated using Cytoscape® v.3.6.1 (SHANNON e colab., 2003). The proteins used to construct this co-expression network were: GR2; FTRA1-2; FTRB; NTRC; TRXs m1-4, f1-2, y1-2, x, and z; Srx; tAPX, sAPX; GPX1 and 7; GRX 12, 14, and 16; 2-Cys-Prx A-B, PrxQ, and PrxII-E. Detailed information regarding the proteins of this network are described in the supplemental Table S1.

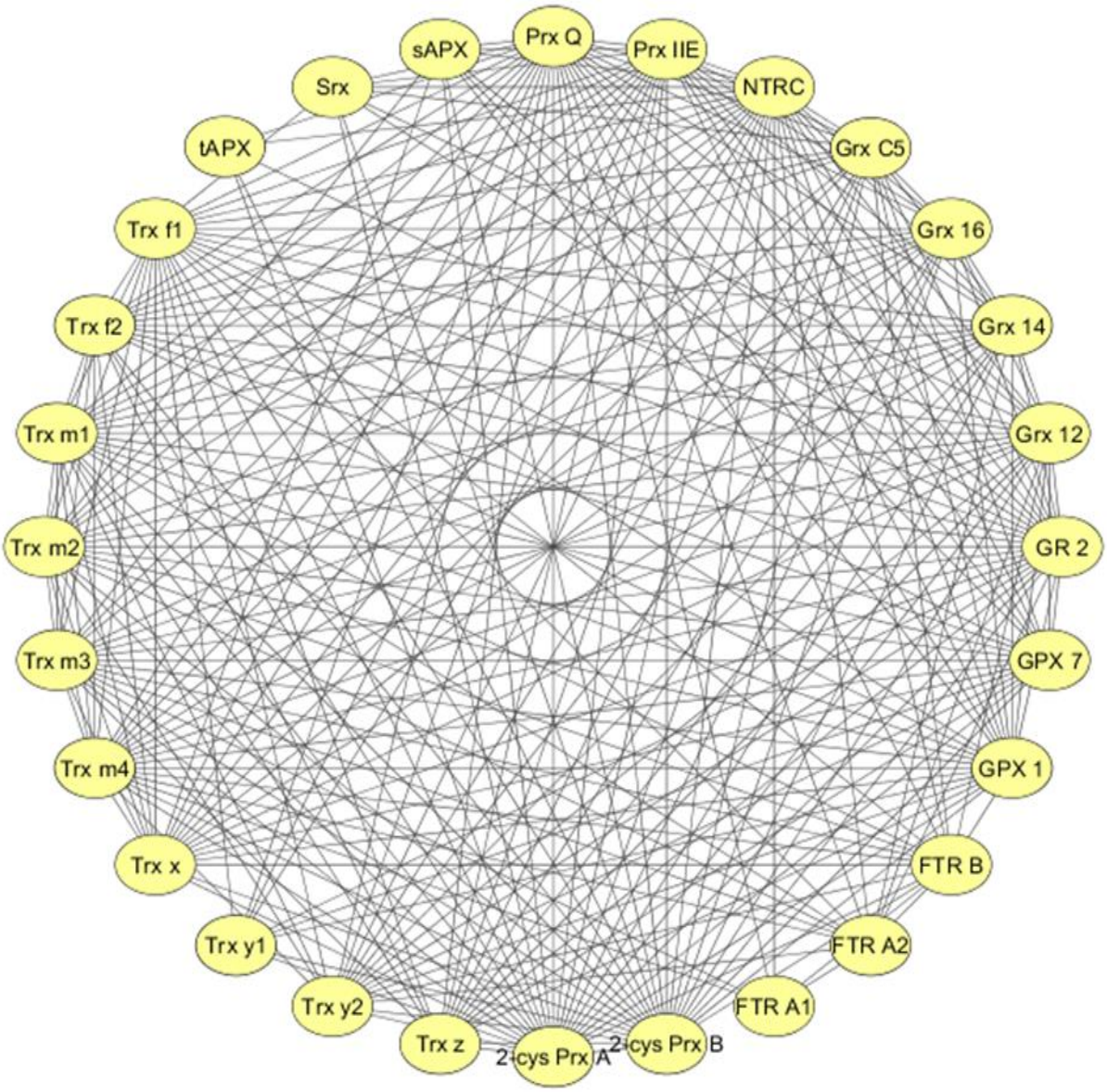

Figure 4 - Protein-protein interaction network between the main components of the chloroplastic redox network. The nodes (enzymes) are connected by a link when their proteins interact to each other according to the Arabidopsis interactome (ARABIDOPSIS INTERACTOME MAPPING CONSORTIUM, 2011). The color of the nodes represents different groups of proteins: Light blue, transcription factors; dark blue, reductases; light brown, peroxidases; green fluorescent, redoxins; light green, other proteins. Red edges represent protein-protein interaction or regulation already observed by in vitro or in vivo studies. Abbreviations and the identification of the proteins of this network are described in the supplemental table S1.

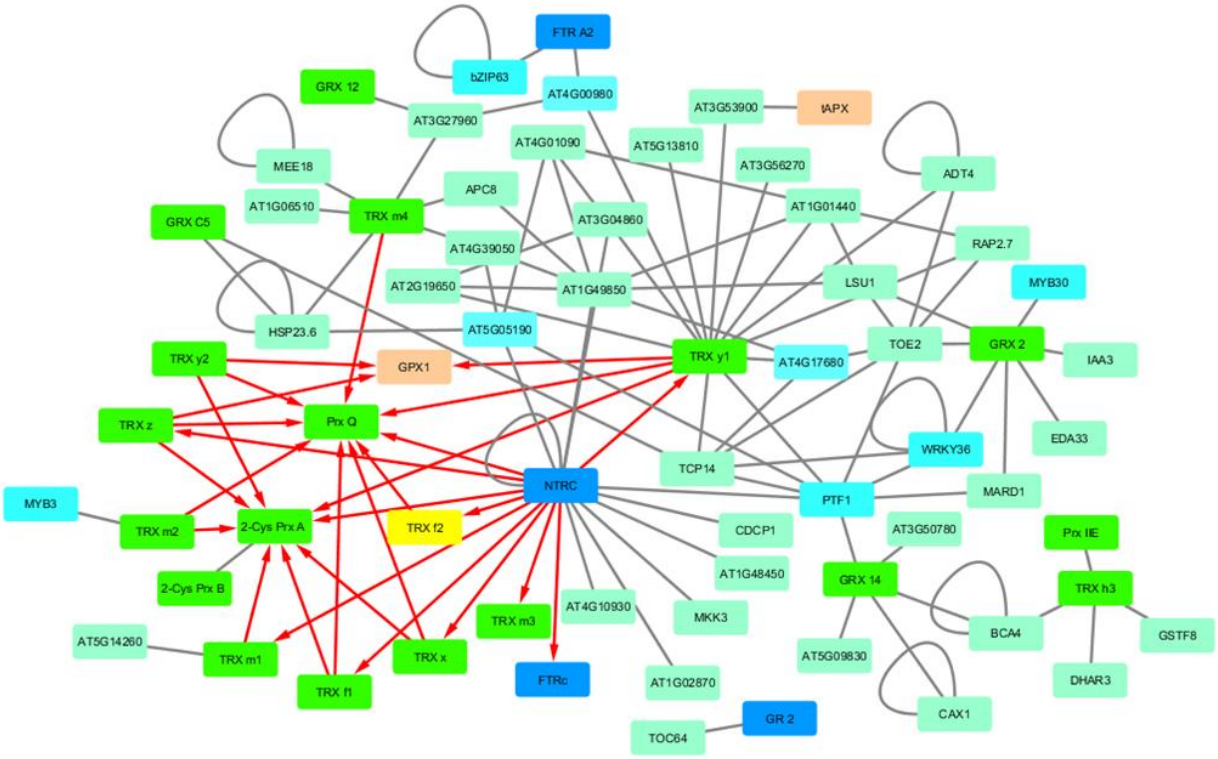

Figure 5 - **Protein-protein interaction network.** The nodes (enzymes) are connected by a link when their proteins interact to each other according to the Arabidopsis interactome (ARABIDOPSIS INTERACTOME MAPPING CONSORTIUM, 2011). Blue nodes are key transcription factors that interact directly or indirectly with NTRC and/or Trx y1.

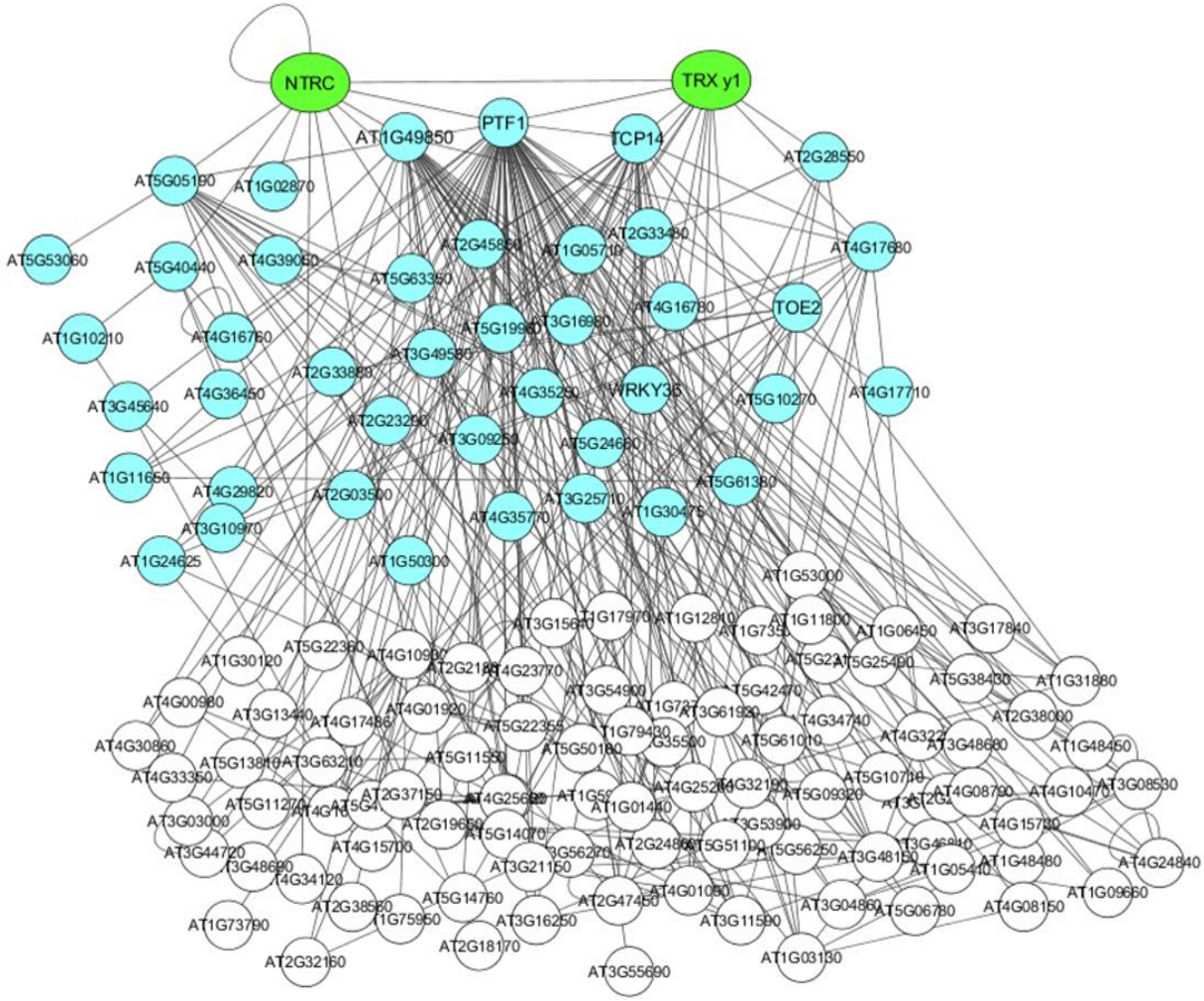

Figure 6 - **Trx h3 protein-protein interaction network.** The nodes (enzymes) are connected by a link when their proteins interact to each other according to the Arabidopsis interactome (ARABIDOPSIS INTERACTOME MAPPING CONSORTIUM, 2011). Green and yellow nodes denote chloroplastic and cytosolic enzymes, respectively. Proteins identified with asterisks (*) are located to both chloroplast and cytosol. Abbreviations and the identification of the proteins of this network are described in the supplemental table S2.

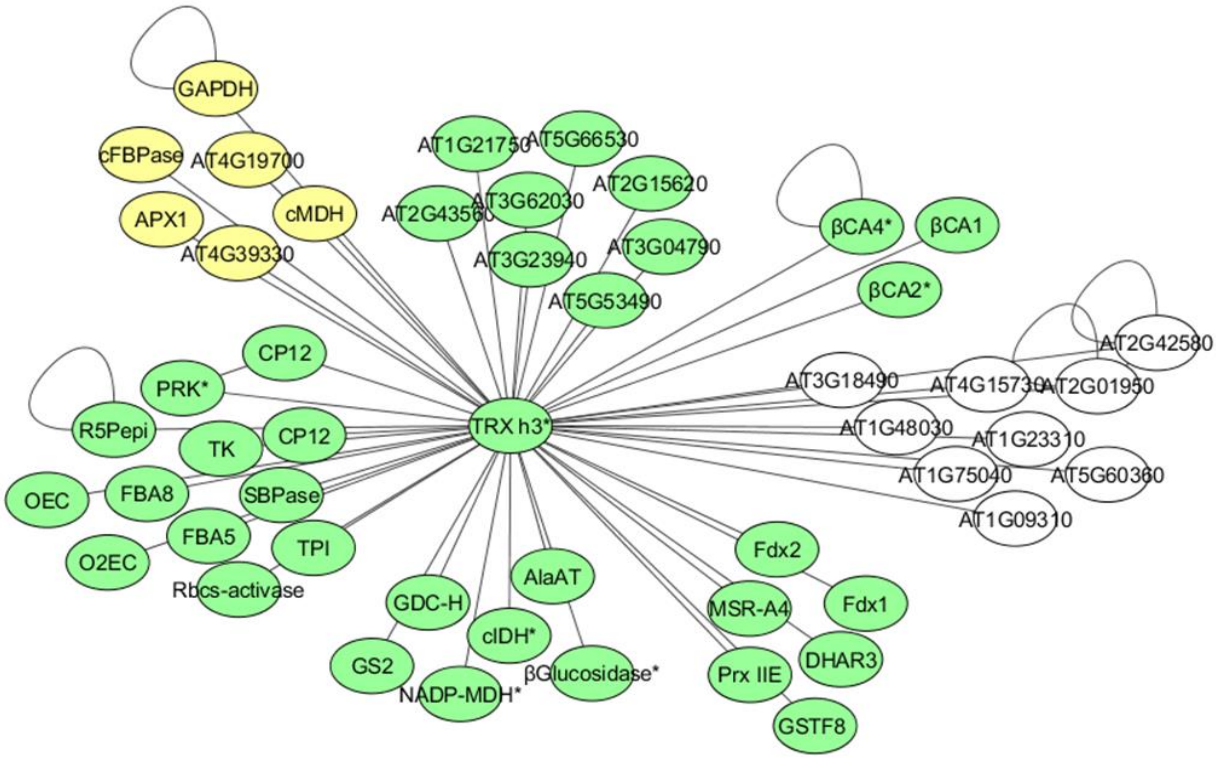

Table 1 - Chloroplastic redox proteins

Arabidopsis Gene ID	Abbreviation	Protein Name	Protein location
AT1G03680	Trx *m1*	Thioredoxin m1 isoform	Chloroplast
AT4G03520	Trx *m2*	Thioredoxin m2 isoform	Chloroplast
AT2G15570	Trx *m3*	Thioredoxin m3 isoform	Chloroplast
AT3G15360	Trx *m4*	Thioredoxin m4 isoform	Chloroplast
AT5G42980.1	Trx *h3*	Thioredoxin h3	Mitochondrion/Cytoplasm/ Chloroplast
AT3G02730	Trx *f1*	Thioredoxin f1 isoform	Chloroplast
AT5G16400	Trx *f2*	Thioredoxin f2 isoform	Chloroplast
AT1G76760	Trx *y1*	Thoredoxin y1 isoform	Chloroplast
AT1G43560	Trx *y2*	Thioredoxin y2 isoform	Chloroplast
AT1G50320	Trx *x*	Thioredoxin type x	Chloroplast
AT3G06730	Trx *z*	Thioredoxin type z	Chloroplast
AT4G09010	APX 4	Ascorbate Peroxidase isoform 4	Lumen
AT4G08390	sAPX	stroma Ascorbate Peroxidase	Stroma
AT1G77490	tAPX	Thylakoid Ascorbate Peroxidase	Thylakoid
At3g54660	GR2	Glutathione Reductase isoform 2	Chloroplast
AT2G25080	GPX 1	Glutathione Peroxidase isoform 1	Chloroplast
AT4G31870	GPX 7	Glutathione Peroxidase isoform 7	Chloroplast
AT2G20270	GPX 12	Glutaredoxin 12	Chloroplast
AT1G31170	SRX	Sulfiredoxin	Chloroplast
AT3G11630	2-Cys Prx A	2-Cys Peroxiredoxin A	Chloroplast
AT5G06290	2-Cys Prx B	2-Cys Peroxiredoxin B	Chloroplast/Mitochondrion
AT3G26060	PrxQ	Peroxiredoxin Q	Chloroplast
AT3G52960	PrxIIE	Peroxiredoxin-II-E,	Chloroplast
AT3G54900	GRX 14	Glutaredoxin 14	Chloroplast
AT2G38270	GRX 16	Glutaredoxin 16	Chloroplast
AT2G41680	NTRC	NADPH Thioredoxin Reductase	Chloroplast
AT5G23440	FTR A1	Ferredoxin/Thioredoxin Reductase Subunit A1	Chloroplast
AT5G08410	FTR A2	Ferredoxin/Thioredoxin Reductase Subunit A2	Chloroplast
AT2G04700	FTR B	Ferredoxin/Thioredoxin Reductase Subunit B	Chloroplast

Table 2 - List of mutants that may be characterized in the near future in order to fulfill the gap of our knowledge regarding the function of specific genes and the complementarity among the components of the redox network.

Mutants	**Compartments involved**	**Possible outputs**
Thioredoxins		
trxh2	Cytosol and mitochondria	The *trxh2* is an uncharacterized mutant. Functional genomic approaches should be applied to investigate the function of TRX *h2*, which is located at both cytosol and mitochondria (MENG, Ling e colab., 2010).
trxh3	Cytosol and chloroplast*	If confirmed that this TRX is also located at the chloroplast, the characterization of this mutant may provide which proteins are target of this enzyme and what it is the role of TRX *h3* for chloroplast development and photosynthetic activity. Given that both carbonic anhydrases βCA1 and βCA4 interact with TRX h3, could this TRX regulate both βCA1 and βCA4 and thus the stomatal response to CO_2?
trxh9	Plasma membrane	The mutation in TRX *h9* is lethal for Arabidopsis (MENG, Ling e colab., 2010), although the reasons for this essentiality remains unclear. Further characterization of this mutant may bring important information whether other redox systems are also involved in this phenotype.
trxh1 trxh2	Cytosol	It seems that both cytosolic fumarase and malate dehydrogenase are positively regulated by TRX *h2* and TRX *h1*, respectively (DALOSO e colab., 2015b; HARA e colab., 2006). The characterization of this double mutant will show whether cytosolic fumarase and malate dehydrogenase can be activated by other TRXs or other redox components and what it is the impact of TRX *h2* and TRX *h1* double mutation to plant growth, especially under stress conditions.
trxo1 trx02 trxh2	Mitochondria	This triple mutant lacks all mitochondrial TRX activity and has never been characterized. Mutation in mitochondrial TRX is lethal for mammalian cells (NONN e colab., 2003). The questions to be addressed

		by the characterization of this triple mutant are: Is the plant mitochondrial TRX system essential for plant growth? Are the components of plant mitochondrial TRX system redundant? Could mitochondrial Grxs compensate the absence of the entire mitochondrial TRX system?
trxy1 ntrc	Chloroplast	These enzymes were characterized as hub-like nodes in the chloroplastic redox network. What is the effect of this double mutation for the entire chloroplastic redox network? The characterization of this double mutant may help to answer this important question.
Thioredoxin reductases		
ntra ntrb ntrc	Chloroplast, cytosol and mitochondria	NTR absence is lethal for mammalian cells (CONRAD e colab., 2004). Surprisingly, neither NTRC nor NTRA-NTRB double mutation are lethal. The characterization of the triple *ntra ntrb ntrc* mutant will answer whether plant NTRs are essential for plants. If not, which redox components can compensate the absence of these enzymes?
Glutathione metabolism		
gr1 gr2	Chloroplast, cytosol and mitochondria.	GR1 and GR2 encode glutathione reductases located at the cytosol and at both chloroplast and mitochondria. Mutation in genes of other reductases such as NTRC and NTRA:NTRB has severe consequences for plant growth. The characterization of *gr1 gr2* double mutant may demonstrate the importance of this reductase system for plants.
ghr1 ghr4	Chloroplast	It was suggested that GHRs are central to the regulation of the quinone redox state (Lallement *et al*. 2014). Photochemistry measurements in single or double *ghr1* and *ghr4* mutants may prove if this glutathione transferase can regulate plastoquinone redox state. No characterization of these mutants was performed so far.
Peroxidases		
gpx5	Plasma membrane	While the other seven GPX isoforms are relatively well characterized in Arabidopsis, not much is known about AtGPX5.
Cross mutants		

ntrc gr2 *ftra ftrb gr2*		The complementarity among NTRC and FTR has been recently demonstrated (YOSHIDA; HISABORI, 2016b). However, whether GR2 can also compensate the absence of the other chloroplastic reductases remains to be determined.
gpx3 rcd1	Secretory pathway, cytosol and nucleus	The radical induced cell death protein 1 (RCD1) supports regulation of genes encoding chloroplast antioxidant enzymes and glutathione biosynthesis (Hiltscher *et al*. 2014). AtGPX3 interacts with RCD1 (Miao *et al*. 2006). Studies with *gpx3 rcd1* double mutant would indicate which redox pathways are directly involved with the interaction between these proteins.
gpx3 abi1 *gpx3 abi2*	Secretory pathway, cytosol, nucleus and plasma membrane	Abscisic acid insensitive 1 (ABI1) and 2 (ABI2) are involved with the abscisic acid signaling pathway (Merlot *et al*. 2002). AtGPX3 interacts with ABI1 and stronger with ABI2, leading to stomatal closure via the activation of plasma membrane Ca^{2+} channels (Miao *et al*. 2006). There is no information about *gpx3 abi1* double mutant, while *gpx3 abi2* double mutant seems to be insensitive to ABA (Miao *et al*. 2006). Experiments with *gpx3 abi1* and *gpx3 abi2* double mutants would improve the understanding about ABA signaling mediated by regulation of the redox state.
The chloroplastic location of Trx *h3* is supported by chloroplastic proteomic studies, but its exact location should be confirmed *in vivo*.		

Table 3 -Thioredoxin H3 interactomic

ARABIDOPSIS GENE ID	ABBREVIATION	PROTEIN NAME	PROTEIN LOCATION
AT3G23940	DHAD	DIHYDROXYACID DEHYDRATASE	CHLOROPLAST
AT5G26000	B-GLUCOSIDADE	BETA GLUCOSIDASE 38	CHLOROPLAST
AT5G35630	GS2	GLUTAMINE SYNTHETASE 2	CHLOROPLAST
AT3G60750	TK	TRANSKETOLASE 1	CHLOROPLAST
AT3G04790	----------	EMBRYO DEFECTIVE 3119	CHLOROPLAST
AT5G14740	BCA 2	BETA CARBONIC ANHYDRASE 2,	CHLOROPLAST
AT1G65930	CIDH	CYTOSOLIC NADP+-DEPENDENT ISOCITRATE DEHYDROGENASE	CHLOROPLAST
AT1G17290	ALAAT	ALANINE AMINOTRANSFERASE	CHLOROPLAST
AT1G32060	PRK	PHOSPHORIBULOKINASE	CHLOROPLAST
AT5G53490	TL17	HYLAKOID LUMENAL 17.4 KDA PROTEIN	CHLOROPLAST
AT5G58330	NADP-MDH	NADP-DEPENDENT MALATE DEHYDROGENASE,	CHLOROPLAST
AT2G35370	GDC-H	GLYCINE DECARBOXYLASE COMPLEX H	CHLOROPLAST
AT3G52930	FBA-8	FRUCTOSE-BISPHOSPHATE ALDOLASE 8	CHLOROPLAST
AT2G21170	TPI	PLASTID ISOFORM TRIOSE PHOSPHATE ISOMERASE	CHLOROPLAST
AT2G39730	RCBS-ACTIVASE	RCA, RUBISCO ACTIVASE	CHLOROPLAST
AT5G61410	R5PEPI	D-RIBULOSE-5-PHOSPHATE-3-EPIMERASE	CHLOROPLAST
AT2G43560	----------	FKBP-PEPTIDYL-PROLYL CIS-TRANS ISOMERASE	CHLOROPLAST
AT3G62410	CP12-1	CP12 DOMAIN-CONTAINING PROTEIN 1	CHLOROPLAST
AT3G50820	OEC33	OEC33 COMPLEX OXYGEN	CHLOROPLAST
AT1G21750	ATPDI5	PROTEIN DISULFIDE ISOMERASE 5,	CHLOROPLAST
AT3G62030	CYP20-3	, ATPDIL1-1, CYCLOPHILIN 20-3	CHLOROPLAST
AT2G15620	NR1	FERRODOXIN NITRI REDUCTASE	CHLOROPLAST
AT2G47400	CP12-1	CP12 DOMAIN-CONTAINING PROTEIN 1	CHLOROPLAST
AT5G66530	----------	GALACTOSE MUTAROSE SUPERFAMILY PROTEIN	CHLOROPLAST
AT5G66570	OEC	OEE33, OXYGEN EVOLVING COMPLEX 33 KILODALTON PROTEIN	CHLOROPLAST
AT4G25130	MSRA4	METHIONINE SULFOXIDE REDUCTASE A4	CHLOROPLAST

ARABIDOPSIS GENE ID	ABBREVIATION	PROTEIN NAME	PROTEIN LOCATION

AT4G26530	FBA5	FRUCTOSE-BISPHOSPHATE ALDOLASE 5	CHLOROPLAST
AT1G04410	CMDH	CYTOSOLIC-NAD-DEPENDENT MALATE DEHYDROGENASE 1, CYT-NADMDH	CYTOSOL
AT4G19700	ATILP, BOI	BOTRYTIS SUSCEPTIBLE1 INTERACTOR,	CYTOSOL
AT1G43670	CFBPASE	FRUCTOSE INSENSITIVE 1, FRUCTOSE-1,6-BISPHOSPHATASE	CYTOSOL
AT2G24270	GAPDH	ALDH11A3 GLYCERALDEHYDE 3 PHOSPHATE DEHYDROGENASE	CYTOSOL
AT3G18490	ASPG1	ASPARTIC PROTEASE IN GUARD CELL 1	ENDOPLASM RETICULUM
AT5G60360	SAG2	ALEURAIN-LIKE PROTEASE, ALP, (SENESCENCE GENE)	EXTRACELLULAR SPACE
AT2G01950	BRL2	BRI1-LIKE 2, VASCULAR HIGHWAY 1, VH1	INTEGRAL COMPONENT OF MEMBRANES
AT1G48030	MTLPD1	LIPOAMIDE DEHYDROGENASE 1,	MITOCHONDRION
AT1G23310	AOAT1	ALANINE-2-OXOGLUTARATE AMINOTRANSFERASE	PEROXISOME
AT4G39330	CAD9	, CINNAMYL ALCOHOL DEHYDROGENASE 9	APOPLAST
AT2G42580	TTL3	TTL3	CELL GUARD
AT1G75040	PR5	PATHOGENESIS-RELATED GENE 5, PR-5,	CELL WALL

3 CHARACTERIZATION OF TRX H2 MUTANTS OF *Arabidopsis thaliana* L.

ABSTRACT

Thioredoxins (Trx) are important proteins involved in redox regulation of plant metabolism. *Arabidopsis thaliana* L. has several Trx isoforms divided in different subfamilies according to their subcellular localization. Trx *h2* is found at both cytosol and mitochondria. Previous studies suggest that this Trx can regulate different mitochondrial enzymes *in vitro*. However, knowledge regarding the function of this Trx *in vivo* is very limited. Furthermore, no study has investigated the impact of Trx *h2* mutation for plant metabolism so far. We thus characterized Arabidopsis plants lacking Trx *h2* through physiological and biochemical analyzes. The *trxh2* mutants showed delayed seed germination with no penalties in photosynthesis and plant growth phenotype. Leaf metabolite profile of *trxh2* mutants revealed alteration in key metabolites of photorespiration such as serine, glycine, glycolate and glycerate beyond several other metabolites involved in respiration and amino acid biosynthesis. Furthermore, $NADH/NAD^+$ ratio decreased in the mutants. Bioinformatics analyzes provided enzymes capable to suffer redox regulation *via* Trx *h2*, including proteins from the tricarboxylic acid cycle and (photo)respiration. We validated the predictions regarding glycine decarboxylase subunits and serine methyl aminotransferase by carrying out western blot analysis. The results showed a decrease in the protein abundance of both GDC and SHMT in *trxh2* mutants. Taken together, or results suggest that Trx *h*2 may have an important role in the redox regulation of mitochondrial enzymes.

Key words: Thioredoxin h2, photorespiration, Glycine decarboxylase, Serine Methyl aminotransferase

1. INTRODUCTION

The redox metabolism is governed by reduction and oxidation reactions that control cell catabolism and anabolism in several cellular compartments, such as plastids, peroxisomes, mitochondria, and cytosol (DIETZ, K.-J.; PFANNSCHMIDT, 2011). Redox metabolism is essential for controlling cellular homeostasis in response to environmental changes. This is accomplished by gene expression reprogramming and post-translation modification of target enzymes by thiol redox regulation (GEIGENBERGER; FERNIE, 2014a). Proteins containing cysteine (Cys) residues are possible target of redox regulation (SCHÜRMANN; BUCHANAN, 2008). Cys residues are preferentially oxidized, leading to the formation of disulfide bridge that can be cleaved by the activity of thioredoxins (Trx) generating two free thiol groups (COMELLI; GONZALEZ, 2007). Trxs consist in ancient redox family protein involved in cellular redox regulation (BALMER, Y. e colab., 2004). These proteins are distributed in all organisms from prokaryotes to complex eukaryotes (Meyer *et al.*, 2012). In *Arabidopsis thaliana* L., Trx is divided into seven families (Trx *m1-4*, Trx *f1-2*, Trx *y1-2*, Trx *o1-2*, Trx *x*, Trx *z*, and Trx *h1-9*), encoded by 20 different genes (GEIGENBERGER e colab., 2017; THORMÄHLEN e colab., 2013, 2015) and distributed in different cell compartments. Trxs *m*, *f*, *x*, *y* and *z* isoforms are located in the chloroplast, whilst Trx *o1-2* are mitochondrial and proteins from Trx *h* are distributed in cytosol (Trx *h1,2,3,4,5,7,8*), endoplasmic membrane (Trx *h9*), and mitochondria (Trx *h2*) (BUCHANAN, 2016b). Trx active site presents at least two Cys and a conserved amino acid residue (^{57}WCGPC^{61}) (CHA e colab., 2008). When Trx is inactivated there is a disulfide bridge between these two Cys residues. Once Trx suffers reduction by a Trx reductase enzyme, Trx becomes activated and gain catalyzes power (ARSOVA e colab., 2010). Plant cells possess different Trx reductases that act according to their subcellular localization. Chloroplasts contain two Trx reductases, named Ferrodoxin Thioredoxin Reductase (FTR) and NADPH-dependent Trx Reductase C (NTRC) (MEYER e colab., 2005; SCHÜRMANN; BUCHANAN, 2008; SUORSA e colab., 2016). In contrast, Trx reduction in nucleus, cytosol and mitochondria is mainly accomplished by NTRA and NTRB. NTR proteins are reduced by NADPH or NADH and then are capable to reduce Trxs. Thus, the balance between NAD^+/NADH and $NADP^+$/NADPH is important to regulate NTR enzymatic activity and consequently the redox metabolism, which is essential for plant growth and development (BERNAL-BAYARD e colab., 2014; REICHHELD, Jean Philippe e colab., 2010).

Given the higher number of isoforms found at the chloroplasts compared to other organelles and the intrinsic complexity of the chloroplastic redox metabolism, the function of the NTR/Trx system and the target proteins of this system have been widely studied in this organelle. The targets of the chloroplastic Trx system identified so far are proteins involved in important metabolic pathways such as ADP-Glucose Pyrophosphorylase (AGPase) of starch biosynthesis (THORMÄHLEN e colab., 2013), ATP synthases (CF1-ATPase), Magnesium Chelatase (CHLI) of chlorophyll biosynthesis pathway (BALMER, Y. e colab., 2004; LUO e colab., 2012) and several enzymes from the Calvin-Benson cycle, for example Rubisco Activase, Fructose-1,6-biphosphatase (FBPase), and sedoheptulose-1-7 biphosphatase, (BUCHANAN e colab., 2015; NARANJO e colab., 2016; THORMÄHLEN e colab., 2015). At mitochondrion, Trx *o* has been proposed to regulate different tricarboxylic acid (TCA) cycle related enzymes such as succinate dehydrogenase (SDH), fumarase (FUM), citrate synthase, isocitrate dehydrogenase as well as alternative oxidase (AOX) that function as an alternative pathway of the mitochondrial electron transport chain (Gelhaye *et al.*, 2004b; Montrichard *et al.*, 2009; Ueoka-Nakanishi *et al.*, 2013;). Notably, it was demonstrated that Trx *o1* is a key regulator of the metabolic fluxes through the TCA cycle by deactivating SDH and FUM (Daloso *et al.*, 2015), which provide additional insights into the mechanisms that inhibit respiration in the light (TCHERKEZ e colab., 2012). Despite these advances on how Trxs regulate photosynthesis and respiration, no studies have investigated the role of Trx *h2* protein yet, a Trx found at both mitochondria and cytosol.

Firstly isolated, identified and purificated from Spinach leaves (FLORENCIO e colab., 1988), the Trx *h* family has been characterized as Trx system beyond chloroplast. Trx *h* proteins are found at mitochondrion, endoplasmic reticulum, and cytosol (GELHAYE; ROUHIER; JACQUOT, 2004). Given the high number of Trx *h* isoforms, there is a high redundancy among this gene family, made it difficult to determine the function of each Trx *h* isoforms (BUCHANAN, 2016b; MEYER e colab., 2009, 2012a). The first function attributed to Trx *h* was *in vitro* reduction of amylase trypsin inhibitor enzymes in cereal (KOBREHEL e colab., 1992). This work was important because it provided Trx could indirectly regulate seed enzymes by regulating a specific inhibitor, amylase trypsin. Later, the same function was demonstrated in barely, but at this time, limiting the action of dextrinase (JENSEN e colab., 2012). Establishment of Trx *h* mutants have provided important genetic tools to identify the possible fu

nctions of these Trxs in biological systems. Mutation in wheat Trx *h9* delayed seed germination and decrease in the abudance of associated germination enzymes (Li *et al.*, 2009). In contrast, the overexpression of Trx *h*5 in the endosperm presented a high germination rate and an increase in the solubilization of storage proteins needed for seedling establishment. Moreover, characterization of *trxh1* mutant suggests a possible redox regulation of CDPK 21, a member of Ca^{+2} kinase dependent kinases, thus attributing a redox regulation of Ca^{+2} signaling (UEOKA-NAKANISHI e colab., 2013). Trx *h2* has been suggested to regulates AOX in poplar (GELHAYE; ROUHIER; GÉRARD e colab., 2004) and the TCA cycle enzymes SDH and FUM in Arabidopsis *in vitro* (Daloso *et al.*, 2015). However, it remains unclear whether Trx *h2* can regulate these and other enzymes *in vivo* as well as what is the role of this Trx in the mitochondrial metabolism.

Mitochondria is an essential organelle for eukaryotes, being fundamental for several metabolic pathways such as ATP production, TCA cycle, apoptosis, lipid oxidation, and amino acid biosynthesis (BAILLEUL e colab., 2015; FROMM e colab., 2016; MAXWELL e colab., 2002; ZHU e colab., 2012). Furthermore, plant mitochondria is important for C3 metabolism because inside this organelle are two key reactions involved in the photorespiratory pathway, the glycine decarboxylation and serine synthesis (LAROSA e colab., 2017; TIMM, Stefan e colab., 2015). These reactions are catalyzed by glycine decarboxylase complex (GDC), which is composed of four subunits (H, T, L, and P) and serine methyl aminotransferase (SHMT) (OBATA e colab., 2016; TIMM, S. e colab., 2013; TIMM, S; BAUWE, 2013; TIMM, Stefan e colab., 2012b). These two proteins have been suggested to be target of Trx, according to affinity chromatography studies (BALMER, Y. e colab., 2004; YOSHIDA e colab., 2013). However, redox regulations of GDC and SHMT have yet to be experimentally tested. Here, we have used *silico*, *in vitro*, and *in vivo* approaches to investigate the function of Trx *h2* for plant growth and mitochondrial metabolism under non-stress growth condition. For this, we have isolated and characterized two Arabidopsis T-DNA knockout *trxh2* mutant lines. Our results demonstrated that a disruption in the expression of Trx *h2* substantially alters metabolite profile and decrease the abundance of the (photo)respiratory proteins GDC and SHMT without penalty in photosynthesis and photorespiration rates and plant growth. The results are discussed in the context of Trx *h2*-mediated redox regulation of metabolism.

2. MATERIAL AND METHODS

2.1.Identification of *trxh2* mutants

Homozygous *trxh2-1* (SALK_079516) and *trxh2-2* (SALK_079507) mutants were obtained from SALK bank and identified by Polymerase Chain Reaction (PCR) using specific primers for Trx *h2-1* (Fw-GATAAGGGAGGAGGAGCTTC and Rv-CCTTAACCAATAGTCTTGTG) and Trx *h2-2* (Fw-AATCATCATCGTTGACTTGCC and Rv-ACACATCCACTTAGCGTGAGG) in a combination with the T-DNA left border (Lb-ATTTTGCCGATTTCGGAAC).

2.2.Gene expression analysis by qRT-PCR

Total RNA was isolated using TRIzol reagent (Ambion, Life Technology) according to the manufactures recommendations. The total RNA was treated with DNAse I (RQ1 RNase free, DNase I, Promega, Madison, WI, USA). The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured using a Nanodrop spectrophotometer. Finally, 2 μg of total RNA were reverse transcribed with Superscript II RNase H2 reverse transcriptase (Invitrogen) and oligo (dT) primer according to the manufacture`s recommendations. Quantitative real-time PCR reactions were performed in a 96-well plate with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems Appllera, Darmstadt, Germany), using Power SYBR Green PCR Master Mix according to Piques *et al*., 2009.

The primers used here were designed using the open-source program QuantPrime-qPCR primer designed tool (Arvidsson *et al*., 2008). The relative transcription abundance was normalized using the constitutively expressed gene actin (At2g37620) and GAPDH (At3g04120) calculated using the $2^{\Delta\Delta CT}$ method. The primers used for qRT-PCR were designed using the QuantPrime software with efficiency over 90% (Arvidsson *et al*., 2008). The primers used for quantification of expression were Trxh2-Fw-TGAGCCAAGTCGCGTCCTCCTAAAG and Trxh2-Rv-CCGAGAAATCAACCACCAGCAG. Data analyses were performed as described by (CALDANA e colab., 2007). Five biological replicates were processed for each condition.

2.3.Plant material and growth condition

All *Arabidopsis thaliana* plants used here were of the Colombia ecotype (Col-0) background. The seeds of the three genotypes used (Wild-Type (WT), *trxh2-1* and *trxh2-2*) were sown on standard greenhouse soil (Top substrate hit, ideal for vegetable growth) in plastic pots with a 0.1-L capacity. The trays containing the pots were placed under a 9/15-h (for western blot) or 16/8-h (for metabolites profiles) day/night cycle photoperiod (22°C/16°C) with 50%/65% relative humidity and 120 μmol photons m^{-2} s^{-1} light intensity. For metabolite profile, 6 week old long day grown plants were harvested and the rossete frozen at End of Night (EN) and End of Day (ED). We further collected leaves from 6 and 8 weeks old plants for western blot analysis.

2.4.Germination assay

To characterize the germination rate, the three genotypes (WT, *trxh2-1*, and *trxh2-2*) were grown in MS media (Murashige and Skoog 1962) without sucrose in Petri dishes. The plates containing the seeds (decontamination with alcohol and stored in refrigeration for 2 days to break dormancy) were placed in growth chamber under 22ºC, 10/14 day-night photoperiod, 120 μmol photons m^{-2} s^{-1}, and 60-70% relative humidity. We distributed 30 seeds of each genotype in a single petri dish, thus allowing to growth of the three genotypes at the same Petri dish. We analyzed the germination rate along of seven days of the sowing using four replicates. A second experiment was made to evaluate the influence of mannitol (as goal to simulate osmotic stress) on the germination rate. Based on previous germination assays, we tested the addition of 200 mM of mannitol in the MS media without sucrose.

2.5.Determination of chlorophyll *a* fluorescence parameters

In vivo chlorophyll *a* fluorescence parameters were also estimated using DUAL PAM 100 (Halz, Germany) using fully expanded leaves from WT and both *trxh2* mutants (*trxh2-1* and *trxh2-2)* by the saturation pulse method in light and dark-adapted leaves (SCHREIBER e colab., 1995). The ETRs of PSI and PSII and the induction and relaxation kinetics of NPQ were calculated as described previously (BAKER; ROSENQVIST, 2004; LIMA e colab., 2018). The intensity and duration of the saturation light pulse were 8.000 μmol m^{-2} s^{-1} and 0.7 s. The maximum quantum yield of photosystem II (PSII) ($F_v/F_m=(F_m-F_o)/F_m$) was measured in dark-adapted conditions and the effective quantum yield of PSII ($\Delta F_m'=(F_m'–F_s/F_m'$) measured in

leaves exposed to actinic light at 1.000 µmol m^{-2} s^{-1} PPFD. The photochemical (qP=(F_m'-F_s)-(F_m'-F_o')) and non-photochemical (NPQ=(F_m-F_m')/F_m') quenchings were calculated as described previously (BILGER e colab., 1995). The F_m' and F_o' parameters correspond to the maximum and minimum fluorescence of dark-adapted leaves, respectively; $F_{m'}$ and F_s are the maximum and steady state fluorescence in the light adapted leaves, respectively, and F_0' is the minimum fluorescence after far-red illumination of the previously light-exposed leaves (FLEXAS e colab., 2008; GENTY e colab., 1989).

2.6.Gas exchange analysis

The CO_2 assimilation rate (*A*), stomatal conductance (g_s), intercellular CO_2 partial pressure (Ci) and transpiration rate (*E*) were measured in fully expanded leaves from WT, *trxh2-1* and *trxh2-2* plants using a portable infrared gas analyzer system (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). The internal parameters in the IRGA chamber during gas exchange measurements were 1,000 µmol m^{-2} s^{-1} PPFD, 1.0 ± 0.2 kPa VPD and 38 Pa CO_2, at 26 °C. The *A* was measured in response to changes in PPFD and Ci. The *A*/Ci curve was carried out using 1000, 400, 300, 200, 100, 50, 400, 600, 800, 1200, 1500 µmol of CO_2. The *A*-Ci fitting curves were determined according to models proposed by (LIETH; REYNOLDS, 1987; SHARKEY e colab., 2007), respectively. The stomatal conductance, transpiration, the photorespiration (Pr) were derived from the *A*-Ci curve, the maximum photosynthetic rate (Amax) and the quantum efficiency (a) were calculated from the *A*-PPFD curve. Photorespiration was calculated by Pr=1/12*(ETR at 1000 µmol (4*(A+RD))) according to (YOSHIMURA e colab., 2001)

2.7.Measurement of pyridine nucleotides

Extraction and analysis of pyridine nucleotides NAD, NADP, NADH and NADPH were performed as described previously (LINTALA e colab., 2014). Briefly, 25 mg frozen leaf powder was resuspended in 250 µl 0.1 M KOH (for NADH and NADPH, respectively), or 250 µl 0.1 M $HClO_4$ (NAD^+ and $NADP^+$) and incubated for 10 min on ice. Then, samples were centrifuged at 20,000 *g* for 10 min at 4°C and the supernatant was heated to 95°C for 2 min. pH was adjusted to 8.0 – 8.5 by titration volume 0.2 M Tris (pH 8.4), 0.1 M KOH or 0.2 M Tris (pH 8.4), 0.1 M $HClO_4$. The final detection mix for NAD (H) contained 100 mM Tricine/KOH (pH 9), 4 mM EDTA, 500 mM EtOH, 0.1 mM phenazine ethosulfate (PES), 0.6 mM methylthiazolyldiphenyl-

tetrazolium bromide (MTT), 6 U ml^{-1} alcohol dehydrogenase (ADH). In addition, for NADP(H) the final mix consisted of 100 mM Tricine/KOH (pH 9), 4 mM EDTA, 3 mM glucose 6-phosphate, 0.1 mM PES, 0.6 mM MTT and 6 U ml^{-1} G6PDH. We quantify absorption monitoring at 570 nm at 30°C in a microplate reader (HT3 Anthos Mikrosysteme GmbH, http://www.anthos.de/). To validate the method, small representative amounts (two to threefold the endogenous content) of NAD^+, $NADP^+$, NADH and NADPH were added on the killing plants material $HClO_4$ or KOH. The recovery of these metabolites from Arabidopsis leaves during extraction and assay were (as percentage of the amount added): NAD+, 92 %; NADP+, 98 %; NADH, 79 % and NADPH, 111 %.

2.8.Measurement of hexose-phosphates, triose-phosphates and 3-PGA

The extraction of harvested leaves and the measurement of hexose-phosphates, triose-phosphates and 3-Phosphoglyceric acid (3-PGA) were made as described by (HÄUSLER e colab., 2000) with following modifications. 50 mg of frozen pulverized leaf material was extracted with 0.7 mL 1M ice-cooled perchloric acid and incubated on ice for 15 min. The pH of the supernatant was modified to 6–7 with 5M K_2CO_3, and after removal of the precipitated $KClO_4$, 8–10 mg activated charcoal was added. The metabolic levels of hexose-phosphates and triose-phosphates were measured by enzymatic reactions (NAD(P)H formation) by NAD(P)H fluorescence using the Safire2microplate reader (Tecan GmbH, Crailsheim, Germany) and the Magellan software version 6.2 (Tecan GmbH). The 3-PGA levels were also measured enzymatically, but in the absorption measuring mode at 340 nm using the Anthos reader HT-3 (Anthos Mikrosysteme GmbH). Every individual plant sample was measured with at least two analytical replicates

2.9.Starch and sucrose measurements

The determination of starch and sucrose was made according to Hendriks *et al*. (2003). 20 mg of pulverized leaf material was extracted three times with 250 mL ethanol (twice with 80% EtOH, once with 50% EtOH). After the addition of the first 250 mL ethanol, the samples were heated for 30 min at 90 °C. The supernatants were collected in separate tubes, and the next extraction volume was added to the pellets for repeating the procedure. The combined supernatants of the ethanol extracts were used for the enzymatic sucrose determination described

by (JONES e colab., 1977) 30–50 mL sample was mixed with 200 mL 50 mM HEPES/KOH (pH 7), 5 mM MgCl2 0.8mM NADP, 1.7mM ATP and G6PDH (0.7 U m^{-1}).While the stepwise addition of 2 mL hexokinase (HK) (2,5 U mL^{-1}), 2 mL PGI (5,8 U mL^{-1}) and 4 mL Invertase (2,8 U mL^{-1}) the NADPH production was determined photo metrically at 340 nm. For starch determination, the pellets of the ethanol extraction were dried at 30 °C for 40 min with a vacuum concentrator (Concentrator Plus, Eppendorf AG, Hamburg, Germany), resuspended in 400 mL 0.1M NaOH, then incubated at 95 °C with 1400 rpm shaking for 1 h (Thermomixer comfort, Eppendorf AG) and neutralized with an HCL/sodium-acetate mixture (0.5M HCl + 0.1Macetate/NaOH, pH 4.9). 40 mL of the supernatant was digested overnight at 37 °C with 110 mL amyloglucosidase (3 U mL^{-1}), a-amylase (4 U mL^{-1}) and 50mM acetate/NaOH (pH 4.9). 30–50 mL of the digested supernatant was mixed with 160 mL 0.1M HEPES/3mM MgCl2 (pH 7), 3 mM ATP, 1.4mM NADP and G6PDH (3.4 U mL-1).To assess the glucose content photometrically,1 mL HK (450 U mL-1) was added. The determination of NADPH at 340 nm was done with the Anthos reader HT-3 (Anthos Mikrosysteme GmbH, Krefeld, Germany). Every individual plant sample was measured with at least two analytical replicates

2.10. Determination of metabolite profile

The whole rosettes were collected in two different times; End of Night (EN) and End of Day (ED) from the three genotypes (WT, *trxh2-1, and trxh2-2*), frozen with liquid nitrogen and stored at -80°C until analysis. Metabolite extraction was carried out by a rapid grinding in liquid nitrogen and addition of appropriated extraction buffer. The primary metabolite profiling was conduct by chromatography coupled with mass spectrometry (GC–MS) protocol as described (LISEC e colab., 2006). Chromatograms and mass spectra were evaluated by using Chroma TOF 1.0 (Leco, http://www.leco.com/) and TAGFINDER 4.0 software (LUEDEMANN e colab., 2008). The amounts of metabolites were evaluated by normalization of signal intensity for ribitol, which was added as an internal standard during the extraction and fresh weight. To a better view, was made a Heatmap for all metabolic successful annotated through Multi Experiment Viewer program. Relative log2-transformed values of signal intensities were normalized with respect to the mean response calculated for the wild type control at end of the night.

2.11. Protein extraction

Total soluble protein extracts were obtained from fresh leaves of *Arabidopsis thaliana* with 6 and 8 weeks of age. It was collected 150 mg of fresh weight, which was ground to powder using with a mortar with liquid nitrogen. Then, we added 1 ml phosphate buffer (100 mM) and 1% of EDTA. After, the extracts were centrifuged at 14,000 *g* for 10 min at 4 °C. Supernatants were collected and the pellets were discarded. The extracts were frozen and stored in -80^{o} freezer.

2.12. Protein immunoblot assay

Total leaf proteins were extracted and separated by SDS-Page with 12,5 % main gel acrylamide concentration (DIEZEL e colab., 1972). 20 ug of protein was added in acrylamide gel The amount of protein was previously quantified according to the Bradford method (BRADFORD, 1976). After the separation by SDS-PAGE the proteins were transferred to a nitrocellulose membrane according (TOWBIN e colab., 1979). The nitrocellulose membranes after transference were incubated with skinned milk to block unspecific protein binding for 3 hours. The protein content was quantified using monoclonal monospecific antibodies for all subunits of Glycine Decarboxylase (GDC-P, GDC-L, GDC-H, and GDC-T), and SHMT following the manufacturer dilution: GDC-P 1:2000, GDC-L 1:2000, GDC-H 1:3000,GDC-T 1:200, and SHMT 1: 1000). After the skinned milk incubation, the membranes were washed with TBS buffer, and then incubated with the primary antibodies cited above overnight (12 hours). Posteriorly, the membranes were washed with TBS buffer and incubated with secondary antibody (Sigma secondary antibody anti-rabbit, dilution 1:20000). Finally, the blots were revealed by BCIP/NBT.

2.13. Bioinformatics analyzes

After the metabolomic analysis, we used several bioinformatics approaches to raise hypothesis in which proteins are possibly regulated by Trx *h2*. We firstly identified where and when Trx *h2* gene is expressed, then we searched for the genes that are co-expressed with Trx *h*2 gene. After that, we start looking for conserved Cys residues in possible Trx target proteins. Once we encountered the conserved Cys residues at the protein sequences, we predicted the disulfide

bridge in the proteins that own two or more Cys residues in their amino acid sequences. Finally, we used the protein modeling to view the disulfide bridge in 3D models. All these steps are described in detail below. These analyses together with the metabolomics results provided a list of possible proteins and pathways that could be regulated by Trx *h2*.

2.13.1. Trx *h2* transcript level in the development of *Arabidopsis thaliana*

Transcriptome database have provided a huge amount of information about the transcript level along in plant development. To explore the different transcript level of Trx, we used the eFP Browser platform (TOUFIGHI e colab., 2005) to evaluate the Trx *h*2 transcript level along of development of Arabidopsis, and which are the tissues that these transcripts have a high or less accumulation at the different plant life stages.

2.13.2. Trx *h2* gene co-expression network

Gene co-expression analysis provides evidence in which genes are co expressed with the gene sequence or number assention gene input. To do that, we used the program String (SZKLARCZYK e colab., 2017) and the Trx *h*2 AGI code (AT5G39950) to identify the genes that are co expressed with Trx *h*2.

2.13.3. Protein-protein interaction analysis

´Protein-protein interaction network database has been used as great ally to identify possible Trx targets. This database contains physical interactions between proteins through bimolecular fluorescence complementation assay, affinity chromatographic, and X-ray assays (ARABIDOPSIS INTERACTOME MAPPING CONSORTIUM, 2011). To show the proteins that interact with Trx *h* isoforms, we used AGI code from all Trx *h* isoforms (BERARDINI e colab., 2015). We used Cytoscape 3-60 program (SHANNON e colab., 2003) to create a Trx *h* isoform protein network to demonstrate which proteins have already been demonstrate to have physical interaction with Trx *h* subfamily.

2.13.4. Alignments of amino acid sequences

Taking into account that Trx *h2* is also found in mitochondria and based in our metabolomic results and in recent evidences showing that TCA cycle enzymes are regulated by

Trx *o1*, we have investigated possible Trx *h2* targets located in mitochondria. First, we searched for proteins that own at least 2 Cys residues in their amino acid sequence. These sequences were collected in Nacional Bank Information Biotechnology (NCBI). Then, it was made alignments with these proteins' sequences through Clustal w omega (https://www.ebi.ac.uk/research). We adopt two different alignments strategies. The first had the objective to provide the conserved Cys residue in different species from different kingdoms, from prokaryotic till most complex eukaryotic organisms. In the second, we used only plant sequences. We ran a Blast-P protein for the sequence of the protein chosen, using Arabidopsis sequence as input (MADDEN, 2002). Amino acid sequences were analyzed using Gene doc program.

2.13.5. Prediction of disulfide bridge formation in amino acid sequences

To predict possible disulfide bridge formation in possible Trx *h2* target proteins, we used DIANNA 1,1 web server (FERRÈ; CLOTE, 2005). All the input protein sequences were from *Arabidopsis thaliana* (Table I).

2.13.6. Modelling *Arabidopsis thaliana* proteins

To confirm the presence of disulfide bond we built 3D protein models with the program switch-modelling protein (https://swissmodel.expasy.org/). The evaluation of the model follow the validation from the program, in which the program classifies the model analyzing the GMQE (Global Model Quality Estimation), it is quality estimation which combines properties from the target-template alignment and the template search method. The resulting GMQE score is expressed as a number between 0 and 1, reflecting the expected accuracy of a model built with that alignment and template. Higher numbers indicate higher reliability. Another parameter to evaluate the model chosen was QMEAN (BENKERT e colab., 2008)which is a composite scoring function based on different geometrical properties and provide both global (*i.e.* for the entire structure) and local (*i.e.* per residue) absolute quality estimates on the basis of one single model. The QMEAN Z-score provides an estimate of the 'degree of nativeness' of the structural features observed in the model and indicates whether the model is of comparable quality to experimental structures. Higher QMEAN Z-scores indicates better agreement between the model structure and experimental structures of similar size. Scores of -4.0 or below is an indication of

models with very low quality; this is also highlighted by a change of the "thumbs-up" symbol to a "thumbs-down" symbol next to the score.

2.14. Statistical analysis

The Student's *t* test was used to compare WT with the mutants at 5% of significance level ($P < 0.05$). All statistical analyses were performed using the algorithm embedded into Sigma Plot® program.

3. RESULTS

3.1.Arabidopsis *trxh2* mutant exhibited unaltered growth phenotype

Given the known importance of Trxs for plant growth and development, we investigated whether mutations in Trx *h*2 affected plant growth. It was used two independent T-DNA insertion mutant lines, SALK_079516 (*trxh2-1)* and SALK_079507 (*trxh2-2*) (Salk collection, http://www.Arabidopsis.org), with two T-DNA insertion mutations in the second intron and in the third exon of Trx *h*2 gene (At5g39950), respectively. The absence of Trx *h*2 transcripts in leaves of the mutants was confirmed by quantitative reverse transcription PCR analysis, which revealed that Trx *h*2 expression was strongly reduced in both knockout lines (Supplement Figure 1). Despite the reduced Trx *h*2 expression, no visible growth phenotype alteration was observed in the mutant lines on their vegetative stage along of 8 weeks (Figure 1). Previous analysis confirmed that biomass accumulation was unaltered in *trxh2* mutants (Pereira, 2017).

3.2.The lack of Trx *h2* delayed seed germination

Trx *h* proteins have been widely recognized as important regulators of seed germination (HÄGGLUND e colab., 2016). We thus characterized the germination rate of WT and *trxh2* seeds along of seven days. The *trxh2-1* and *trxh2-2* mutants presented a delay in the germination rate compared with WT (Figure 2a). The germination of mutant seeds started only after the third day of the experiment, which leads to a decreased germination velocity (Figure 2a-b). We further explored the influence of osmotic stress on germination rate by sowing seeds under 200 mM of mannitol. Both WT and mutant seeds only germinated after the second day. However, *trxh2-1* and *trxh2-2* showed a lower germination rate than WT (Figure 2c). Despite the lower germination rate, WT, *trxh2-1,* and *trxh2-2* present the same germination velocity (Figure 2 c-d).

3.3.The Trx *h2* mutation does not alter photochemical and gas exchange parameters

Although Trx *h2* is a non-plastid protein, several evidences suggest that alteration of genes from cytosolic redox metabolism can impair photosynthesis (RAHANTANIAINA e colab., 2017). We thus decided to investigate whether the lacking Trx *h*2 impact photosynthesis in Arabidopsis. We analyzed photochemical and gas exchange parameters using DUAL PAM 100 (Halz, Germany) and an infra-red gas analyzer (IRGA LI-6400XT, LI-COR, Lincoln, NE, USA). The mutants exhibited no significance changes in electron transport rate (ETRI and ETRII) (Figure 3). Furthermore, no differences were observed in Fv/Fm and NPQ parameters in both *trxh2* mutants (Supplement Figure 2, and figure 4). Similarly, both *trxh2-1 and trxh2-2* mutant lines did not present significant difference in stomatal conductance (g_s) and in the rate of CO_2 fixation (*A*), photorespiration (Pr), and transpiration rate (*E*) compared to WT (Figures 5-6). Despite these lack of differences between the mutant lines and WT, it is important to highlight that both *trxh2* mutant lines presented a decrease in dark respiration (Rd) (WT: 1.48±0.01; *trxh2-1*: 1.18±0.016; *trxh2-2:* 1.22±0.03) (Pereira, 2017).

3.4.The Trx *h*2 is involved in redox balance of pyridine nucleotides

Mitochondria and chloroplast are organelles essential for the metabolism of pyridine nucleotides (NAD^+, $NADP^+$, NADH, and NADPH), given that within these organelles occur several reactions that use NAD(H), NADP(H), and ATP as enzymatic cofactors. We investigated the influence of Trx *h*2 absence on pyridines abundance at the end of the day (ED) and end of the night (EN). The results showed no statiscal difference relation WT in NADH and NADPH abundance in both mutants. In contrast, *trxh2-2* present a decrease in $NADP^+$ and increase in NAD^+ abundance at ED. Interestingly, the NADH/NAD^+ ratio decreased in both mutants at ED (Figure 7).

3.5.Leaf metabolite profile of *trxh2* mutants

To investigate whether Trx *h2* mutation alters primary metabolism, we analyzed the metabolite profile of leaves harvested in ED and EN using a well-established Gas chromatography-time of flight- mass spectrometry (GC-TOF-MS) platform (LISEC e colab., 2006). The results revealed several metabolic changes in the mutants. The *trxh2-1* presented

significant increase in β-alanine, glycine, isoleucine, leucine, ornithine, serine, tyrosine, valine, threonine GABA, gulonic acid, tartaric acid, glucose, fructose, galactinol, raffinose, and spermidine in EN. By contrast, the level of alanine, pyruvate, cellobiose, turanose showed a significant decrease in the EN. In the ED, the mutant *trxh2-1* presented a higher level of glutamine, isoleucine, lysine, serine, tyrosine, GABA, malate, phosphoric acid, glucose, fructose, and galactinol, while the metabolite level of alanine, pyruvate, and cellobiose decreased. In another hand, *trxh2-2* presented a decrease in metabolite level of alanine, arginine, glutamine, ornithine, dehydroascorbate, glycolate, pyruvate, cinnamic acid, cellobiose, ribose-5-phosphate, turanose, guanidine, and putrescine. Whilst the metabolite level of serine, threonine, GABA, malate, glucose, fructose increased in *trxh2-2* at EN, *trxh2-2* presented an increse in isoleucine, lysine, serine, GABA, pyruvate, glucose, fructose, turanose, galactinol, and a decreased alanine and putrescine metabolite levels at the ED (Figure 8 and 9).

Beyond metabolite profile *via* GC-MS analysis, it was also investigated whether the absence of Trx *h*2 impacts starch and sugar metabolism. The starch content decreased only in the *trxh2-2* mutant line. Interestingly, both mutants accumulated more glucose in EN. In addition, the metabolite levels of glucose-6-phophaste significant decreased in both mutants (Figure 9). Taken together, these results suggest that Trx *h2* mutation alters the leaf metabolite profile. Notably, it was observed that several metabolites of photorespiration such as serine, glycine, pyruvate, glycolate, and glycerate were altered in *trxh2* mutant lines.

3.6.*In silico* analysis provides possible Trx *h2* target proteins

We first investigated Trx *h*2 expression along Arabidopsis development using *in silico* platforms. Trx *h2* is expressed throughout the whole plant, but with higher expression in leaves at 6 and 8 weeks, transition reproducible Arabidopsis's development stage specifically at flower stage 15 week, and Roots (Supplement Figure 3). We next have investigated which genes are co-expressed with Trx *h*2. The data is based on experimental and gene annotation analyzes. The results showed a correlation between Trx *h*2 with NTR A, NTR B, Peroxiredoxins Q (PRX Q), glutamine synthetize 1 and 2, and PRX (Supplement Figure 6). The results suggest that Trx *h*2 could be involved in nitrogen metabolism, once glutamine synthetize is a fundamental enzyme in ammonium assimilation, and participating in many metabolism pathways, for example photorespiration. Another evidence gave by these results is that Trx *h*2 can be involved in the

regulation of Peroxiredoxins (PRX). In fact, it has been proposed an interplay between Trxs and PRXs, in which Trx is capable to reduce some PRX such as PRX Q and Cys-2-PRX (Jeong *et al.*, 2006; Journal, 2013; Puerto-Galán *et al.*, 2015). However, it is important to highlight that transcript level not always correlate with protein expression. Thus, we next carried out protein-protein interaction analysis to investigate which proteins can physically interact with Trx *h2*. Protein-protein interaction analysis offers important information of experimental interaction between proteins. These interactions are from experimental assays such as BiFC, X-ray, and affinity chromatography. Differently of others Trxs already characterized, Trx *h*2 only presented 6 protein nodes interaction. Trx *h*2 interactome demonstrated that Trx *h*2 interacts with yeast and Arabidopsis proteins such as methione sulforedoxin reductase (MSR), cytoplasmatic thiol peroxidase (TSA1), CDC33 (mRNA cap involved in cellular cycle), and thioredoxin disulfide reductase of yeast (TRR1) (Supplement Figure 4). It is important to highlight that all yeast proteins are located in the mitochondria. Unfortunately, we could not correlate our interactomic result with metabolite mutant profiles. However, we elected possible Trx h2 target based with our interactomic result; MSR and CDC33 could be target of Trx *h*2 in Arabidopsis, once these enzymes presented a physical interaction with Trx *h*2 already known in yeast.

3.7.Disulfide bridge prediction on photorespiration and respiration enzymes.

Unfortunately, the gene co-expression and Trx *h*2 interactome analyses did not show any enzyme that could be target of Trx *h2* and that could at least partially explain the results found in the *trxh2* metabolite profile. As previously mentioned, Trx proteins are capable to reduce disulfide bridges. Thus, Trx target protein must have in their amino acid sequence at least two Cys residues to make a disulfide bridge, which seems to be conserved among different organisms (Daloso *et al.*, 2015). We therefore searched for proteins that could possibly form disulfide bridge based in their amino acid sequences. The proteins to be analyzed were chosen according to the results of the metabolite profile. Given that the level of several photorespiratory metabolites such as glycolate, hydroxypyruvate, and 3-phosphoglycerate changed in *trxh2-1* and *trxh2-2*, we decided to investigate the presence of disulfide bridge in photorespiratory and associated enzymes. We collected protein sequences involved in photorespiration (GDC subunits: H, T, P, L, and SHMT), respiration (proteins from the TCA cycle), and nitrogen assimilation (GS and GOGAT). A detailed description of the proteins is found in table I. It was observed a conserved

Cys in all proteins cited above, excepted in SHMT that was observed only one Cys residue conserved in all species studied, although Arabidopsis contains more Cys in its amino acid sequence. However, this fact is not a confirmation of the formation of disulfide bridge, once the distance between two Cys residue must be lower than 3,65 A. To better predict the presence of disulfide bridge, we used the Dianna program (FERRÈ; CLOTE, 2005). This program predicts the possible formation of disulfide bridge at a specific protein sequence input and generated a score from 0 (worse prediction) to 1 (better prediction). Three GDC subunits H, T, and P present scores over 0.99, showing the high probability of these Cys residues made a disulfide bridge, while the subunit L presented score lower than 0,02. SHMT showed the possible formation of three amino acid Cys residues, all the prediction presented scores over 0,99. Regarding the TCA cycle enzymes, fumarase 2, succinate dehydrogenase (SDH), and aconitase presented scores over 0.99. In another hand, isocitrate dehydrogenase (IDH) presented score of 0.67, while citrate synthase and malate dehydrogenase own score below 0.02. Furthermore, our results showed the presence of disulfide bridge on glycolate oxidase (score was 0.999), differently of hydroxypyruvate reductase and phosphoglycolate phosphatase, whose score were below 0.02. For glutamine synthase (GS), glutamate oxoglutarate aminotransferase (GoGAT), and alanine aminotransferase we observed scores of below 0.02, 0.98 and 0.678, respectively (Table II). Taken together, these results predicted which proteins form disulfide bridge and consequently can be regulated by Trx *h*2. Notably, three subunits of glycine decarboxylase complex and SHMT owns high score being potential candidates to suffer redox regulation by Trx. In respiration enzymes only Fum 2, SDH, aconitase, IDH demonstrated a possible target via Trx. This data corroborates previous results in which these TCA cycle enzymes have been demonstrated to be regulated by Trx *o*1 and/or Trx *h*2 *in vitro* (DALOSO e colab., 2015a; EPRINTSEV e colab., 2016; FERRARIO-MERY e colab., 2002)

3.8.Modeling enzymes from (photo)respiration

Our disulfide bridge results provide some clues on which proteins might be Trx target. To investigate the formation of disulfide bridge in the native protein, we create 3D models using the modelling program from Protein Data Bank (PBS). We used Arabidopsis sequences from photorespiration, respiration, and nitrogen metabolism according to our previous results. The quality and validation of the models were based on QMean parameters and similarity sequence

between the models with protein templates from PBS. Only protein models whose similarity sequences were over 30% and Qmean above -4.0 were classified as high quality models (BENKERT e colab., 2008; SONI; MADHUSUDHAN, 2017). Some model protein presented a disulfide bridge on output models, which are represented by a purple sphere. However, other protein models did not show a disulfide bridge. In these cases we added a manual disulfide bridge between the Cys residues, whose scores were above 0.97 (cores generated in Dianna program), representing in yellow the disulfide bridge (BIASINI e colab., 2014; SONI; MADHUSUDHAN, 2017).

Glycine decarboxylase H, alanine aminotransferase, GOGAT, and GS2 presented Qmean below -4.0 and similarity sequence lower than 30%. It was confirmed the presence of disulfide bridge in Fum 2, GDC-P1, citrate synthase, IDH, and AOX (Figure 11, and supplement Figure 6). Despite the disulfide bridge absence on SDH, GDC-T, Fum 1, and aconitase, the high Dianna score between Cys residues and high amino acid sequence similarity made possible the manual disulfide creation (in yellow) (Figure 11, and Table II). In another hand, the hydroxypyruvate reductase, phosphoglycolate phosphatase, and GS owned Qmean or similarity sequence score below -4.0 and 30%, which generated models with a sequence different with Dianna Arabidopsis thaliana protein sequence input, thus made the manual disulfide bridge creation unfeasible.

3.9. Western blot analysis revealed decreased abundance of GDC and SHMT proteins in *trxh2* mutants

Next, we carried out western blot analysis using specific antibodies for proteins that seem to be Trx regulated based in the results of the metabolite profile of *trxh2* mutant lines and the *in silico* bioinformatics analyzes. We evaluated protein abundance of all subunits GDC-complex and SHMT. To quantify the protein expression level, we made a WT protein content gradient (50%, 75%, and 100%). The protein expression in the mutants was thus quantified based on optical density in relation with this gradient. Our results show a significant decrease in protein content in GDC-H, GDC-L and SHMT in both mutants, while GDC-T showed a significant increase in protein expression on both mutant lines but statistical differences were limited to the *trxh2-2*. The level of GDC-P subunit was also reduced in the mutants, but in this case without statistical differences (Figure 12 and 13).

4. DISCUSSION

4.1.The lack of Trx *h*2 leads to delay on seed germination

The function of Trx *h* subfamily in Arabidopsis seed germination has been widely investigated (SHAHPIRI e colab., 2009). However, due to the high number of Trx isoforms and other compensatory redox mechanisms, the identification of the function of specific Trx *h* isoforms has been a great challenge (BUCHANAN, 2017). Nevertheless, the characterization of *trxh* mutants has attributed a common function in several Trx *h* isoforms. For instance, both *trxh1* and *trxh9* mutants have a delay in seed germination rate, while Trx *h*5 overexpression accelerates germination rate by an increase solubilization of storage proteins needed by the new seedling (Cho *et al.*, 1999; Wong *et al.*, 2004; Alkhalfioui *et al.*, 2007; Meng *et al.*, 2010a; Buchanan, 2017). The first enzyme identified that suffers redox regulation via Trx *h* was amylase/Trypsin inhibitor, which acts on the mobilization of storage carbohydrates required for seed germination (KOBREHEL e colab., 1992). Further evidences suggest that Trx *h* acts in the redox status of storage enzymes in wheat and barley (Kobrehel *et al.*, 1992; Kaminaka *et al.*, 1998; Alkhalfioui *et al.*, 2007; X *et al.*, 2016). Beyond Trx *h* proteins, it seems that the mitochondrial Trx system is also important for seed germination, given that both the *ntra ntrb* double mutant and *trxo1* mutant present a delay in seed germination (Daloso *et al.*, 2015). Interestingly, our results corroborated the same delay in both Trx *h*2 mutant lines (*trxh2-1 and trxh2-2)* in media without sucrose (Figure 2a) or under osmotic stress (Figures 2 c-d). The lack of Trx *h*2 reduced both the rate and the velocity of seed germination (Figures 2a-b). This suggests a role of Trx *h2* in seed germination, which is in agreement with our *in silico* analysis that has demonstrated an increase in Trx *h*2 expression along of seed embryonic development (Supplements Figure 2).Take together, these results suggest that Trx *h*2 may reseemble the role of others Trx *h* proteins in seed germination. Given that seed germination demands a high mitochondria activity and the increase on storage protein mobilization, it is reasonable to hypothesize that Trx *h2* may act in seed germination by regulating mitochondrial enzymes and/or proteins for storage remobilization (HÄGGLUND e colab., 2016). However, further studies are required to identify Trx *h2* targets in seeds.

4.2.Trx *h2* mutation does not alter photosynthesis

The use of mutants have provided important information concerning crosstalk between plant cell organelles, in which the influence of mitochondrial and chloroplastic enzyme perturbation could affect enzymes in different subcellular locations (BLANCO e colab., 2014). The crosstalk regulation between chloroplast and mitochondria is probably the most studied inter organelar system in plants (RAHANTANIAINA e colab., 2017). For example, great attention has been giving to the mitochondrial enzyme alternative oxidase (AOX), which seems to be central player among the mitochondrial components, being important to maintain chloroplast redox homeostasis and photosynthetic rate (Florez-Sarasa *et al.*, 2016b; Del-Saz *et al.*, 2018; Vishwakarma *et al*. 2014; Welchen & Gonzalez 2016; Dahal *et al*. 2017). In the other hand, Arabidopsis mutants lacking proteins related to the cyclic electron flow around photosystem I exhibited higher AOX activity under high light (FLOREZ-SARASA; NOGUCHI e colab., 2016). Trx *h*2 location was firstly described by GFP assay in onion epidermal cells (Meng *et al.*, 2010), which showed that Trx *h*2 is located at cytosol and mitochondria. Taking these evidences into account, we investigated whether the lack of Trx *h2* impair photosynthesis. The results did not show any influence of Trx *h*2 on photochemical parameters (ETRI, ETR II, NPQ, and Fv/Fm) (Figures 3 and 4). Furthermore, the gas exchange analysis in *trxh2* mutant lines also did not show any difference in *A*, g_s, and *E* (Figure 5). This indicates that Trx *h*2 does not alter photosynthesis under non-stress conditions. Despite this lack of effect on photosynthesis, *trxh2* plants have lower dark respiration (Rd) (Pereira 2017), suggesting a role of this Trx in mitochondrial respiration. Similarly, lack of mitochondrial Trx *o1* has been shown to alter the activity of several mitochondrial enzymes (BUCHANAN, 2016b; DALOSO e colab., 2015b) indicating that the mitochondrial Trx system is important in the regulation of the respiration process. However, whether Trx *h2* resemble Trx *o1* in the context of redox regulation of mitochondrial enzymes remain to be determined.

4.3.Trx *h2* mutation decrease leaf NADH/NAD^+ ratio

The role of pyridine nucleotide nicotinamide adenine dinucleotide(s) in redox metabolism has received considerable attention in plants (GAKIÈRE e colab., 2018). NADH and NADPH are important and intrinsic components of redox metabolism, being essential cofactors for several enzymes, contributing to pH cell control, and also as allosteric enzyme regulators from respiratory and photosynthetic pathways (FOYER, Christine H.; NOCTOR, 2013; NUNES-NESI e colab., 2013; PÉTRIACQ e colab., 2016; RASMUSSON e colab., 2008). Dehydrogenases dependent of NAD^+ or $NADP^+$ as enzymatic cofactors are the main source of NADH and NADPH, and are located mainly at chloroplast and mitochondria (BIENFAIT, 1985). In addition, Arabidopsis contains three genes coding NAD kinases (NADk1, 2 and 3), that are responsible to convert NAD into NADP (CHAI e colab., 2006; WALLER e colab., 2010). NAD^+ is a common cofactor of three enzymes of the TCA cycle (IDH, SDH, and MDH), which provides NADH for oxidation in the mitochondrial electron transport chain (mETC) (SCHEIBE e colab., 2005). Beyond being a substrate in the complex I of the mETC, NADH accumulation also inhibits the activity of several mitochondrial dehydrogenases (NOCTOR e colab., 2007). For instance, it has been proposed that pyruvate decarboxylase (PDC) as well as TCA cycle dehydrogenases are inhibited by NADH *in vitro* (MOONEY e colab., 2002). Moreover, changes in NAD^+/NADH ratio *in vivo* could differentially affect enzyme kinetic parameters (Km, K_{cat}, and k_{cat}/Km) (PASCAL e colab., 1990). Thus, it is reasonable to assume that mitochondrial NAD^+/NADH ratio has a major role to modulate the flux through the TCA cycle and mETC (Geigenberger and Fernie, 2014; Nietzel *et al.*, 2017). Our results showed that *trxh2-2* mutant has decreased $NADP^+$ and increased NAD^+ at ED, while NAPDH content decreased only *trxh2-1* at EN (Figure 7). Interestingly, NADH/NAD^+ ratio decreased in both mutants at ED, differently of NADPH/$NADP^+$ ration that decreased only in *trxh2-1* at EN. These results are similar to the facts described in *trxf1* mutant (THORMÄHLEN e colab., 2017), which may, at least partially, explain the decrease in Rd found in the *trxh2* mutant lines. The decrease in NADH/NAD^+ in *trxh2* mutants may be due either by impairment in the activity of TCA cycle dehydrogenases and/or by a lower use of NADH by NTR A and NTR B. The first idea is based in the fact that the TCA cycle dehydrogenases IDH and SDH have already been shown to be Trx regulated (Yoshida and Hisabori, 2014; Daloso *et al.*, 2015) Thus, the lack of Trx *h2* may alter the activity of these

enzymes leading to a new balance of NADH/NAD. Furthermore, it is noteworthy that Trx *h*2 is co-expressed with both NTR A and B (Supplement figure 5), suggesting that the lack of Trx *h*2 may have consequences in NTR expression and consequently in an unbalanced $NADH/NAD^+$ ratio.

4.4.Redox regulation of (photo)respiration mediated by Trx *h2*

Photorespiration is an important process for C3 plants, being marked for the assimilation of O_2 instead of CO_2 by ribulose-1,5-bisphophaste oxygenase/carboxylase and the regeneration of 2-phosphoglycolate in 3-phosphoglycerate (BAUWE e colab., 2010). Photorespiration is a pathway dependent on three organelles, chloroplast, peroxisome, and mitochondria (FERNIE, 2017). Results from affinity chromatography studies suggest that the mitochondrial photorespiratory enzymes GDC-complex and SHMT are Trx targets (Balmer *et al.*, 2004; Yoshida *et al.*, 2015). Taking this into account, we carried out metabolite profile and western blot analysis for different subunits of GDC-complex and SHMT in WT and *trxh2* mutant lines. The metabolite profile showed alteration in the level of serine and glycine, which are substrate and product of these enzymes, respectively (Figure 12). In addition, our bioinformatics analyzes showed a high probability to the formation of disulfide bridge in GDC-complex and SHMT (Tables I-II; Figure 11). Our immune blot results showed a significant decrease in GDC-H and L protein abundance and increase in GDC-T in *trxh2-2*. Moreover, decreased SHMT abundance in both *trxh2* mutant lines was observed (Figures 13 and 14). Interestingly, the level of other two photorespiratory-related metabolites named glycolate and glycerate were also altered in *trxh2* mutants. Despite these evidences at both proteins and metabolite levels, the photorespiration rate estimated by gas exchange analysis did not show significant alteration. The increase in glycine level may be related to the decrease in GDC protein abundance. Furthermore, we cannot exclude the possibility that the lack of Trx *h2* leads to a less activated GDC, in case both GDC-L and GDC-H are regulated by Trx *h*2 as suggested by metabolomic and bioinformatics analysis. Intriguingly, SHMT protein abundance decreased in *trxh2* mutants while serine level increased in both mutants at ED and EN. A similar phenotype was observed in Arabidopsis mutant lacking GDC-H, in which a moderate decrease in SHMT and GDC-P was observed (TIMM, Stefan e colab., 2018). It is noteworthy that GDC activity is inhibited by serine (PETERSON, 1982) and controlled by $NADH/NAD^+$ ratio (BOURGUIGNON e colab., 1988). Moreover, decreased

SHMT protein abundance was observed in Arabidopsis treated with 1 mM of serine (TIMM, S. e colab., 2013). Thus, the decrease in SHMT protein abundance could be a physiological response to the high amount of serine or the altered NADH/NAD^+ ratio in both Trx *h*2 mutants. Furthermore, it has been shown that modification in the GDC-H expression not only changed Gly levels but also leads to a reprogramming of the photorespiratory metabolism. For instance, reduced level of glycerate or glycolate was observed in Arabidopsis GDC-H overexpression plants (Timm *et al*., 2012). In contrast, while GDC-H abundance decrease in *trxh2* mutants, an increase in glycine content at ED and decreased content of glycerate and glycolate was observed. Taken together, our results suggest that the photorespiratory metabolism may be redox regulated via Trx *h*2, in which glycine and serine synthesis and degradation mediated by GDC complex and SHMT may be a key point for the regulation of this pathway. The consequences of GDC regulation are beyond the photorespiration, given that GDC has an important role in NADH production within mitochondria, which has a pivotal role for establishing allosteric regulation of TCA cycle enzymes as well as is an important substrate for mETC (BAUWE e colab., 2010). Taken together, the results from metabolite profile, pyridine nucleotides and western blot analysis suggest that Trx *h2* may regulate respiration possibly by regulating the amount and/or the activity of GDC, SHMT and other TCA cycle enzymes. However, further experiments are needed to demonstrate whether Trx *h2* can regulate GDC-subunits, SHMT and TCA cycle enzymes *in vitro* and *in vivo*.

4 CONCLUDING REMARKS

The characterization of *trxh2* mutants revealed important information regarding the function of this Trx. The lack of Trx *h2* delayed seed germination, resembling the role of other Trx *h* proteins that are pivotal for seed germination and seedling establishment (Hägglund *et al.,* 2016). Further experiments are necessary to identify which enzymes are redox regulated by Trx *h*2 in seeds. Beyond that, our results suggest that Trx *h2* mutation do not impair photosynthesis and plant growth, at least under non-stress conditions. However, respiratory metabolism seems to be directly or indirectly regulated by Trx *h2*. This idea is based in the fact that several metabolites related to the TCA cycle and the NADH/NAD^+ ratio was altered in the *trxh2* mutants. Furthermore, western blot analysis revealed that the abundance of both GDC and SHMT decreased in the mutants. This suggests that these enzymes are regulated by Trx *h2*, although further gel shift analysis are needed to confirm whether these occurs *in vitro* and *in vivo*.

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APPENDIX A – OTHER FIGURES

Figure 7 - Wild type (WT), trxh2, and trxh2-2 on 8 weeks life development. The Arabidopsis thaliana grew in a refrigerated Universidade Federal do Ceará (UFC) room. The growth conditions were 120μmol photon $m^{-2}s^{-1}$, 55% relative humidity, and short-day photoperiod (9h on light/ 15 on the dark).

Figure 8 - a) Germination rate characterization of trxh2 mutants b) Mutants' characterization velocity Germination c) Mutants characterization under 200mM mannitol treatment d) Mutant's characterization germination velocity under 200Mm mannitol treatment. In the first experiment 30 seeds were distributed per genotype at petri dish (repetition, petri dish=4). In the second experiment was investigated the influence of mannitol 200mM on germination rate, in this experiment was distributed 15 seeds per genotype at petri dish (repetition, petri dish=3). The results were evaluated with T Student T-test P <0,05, statistical difference is represented by asterisk. Error bars represent standard error (S.E)

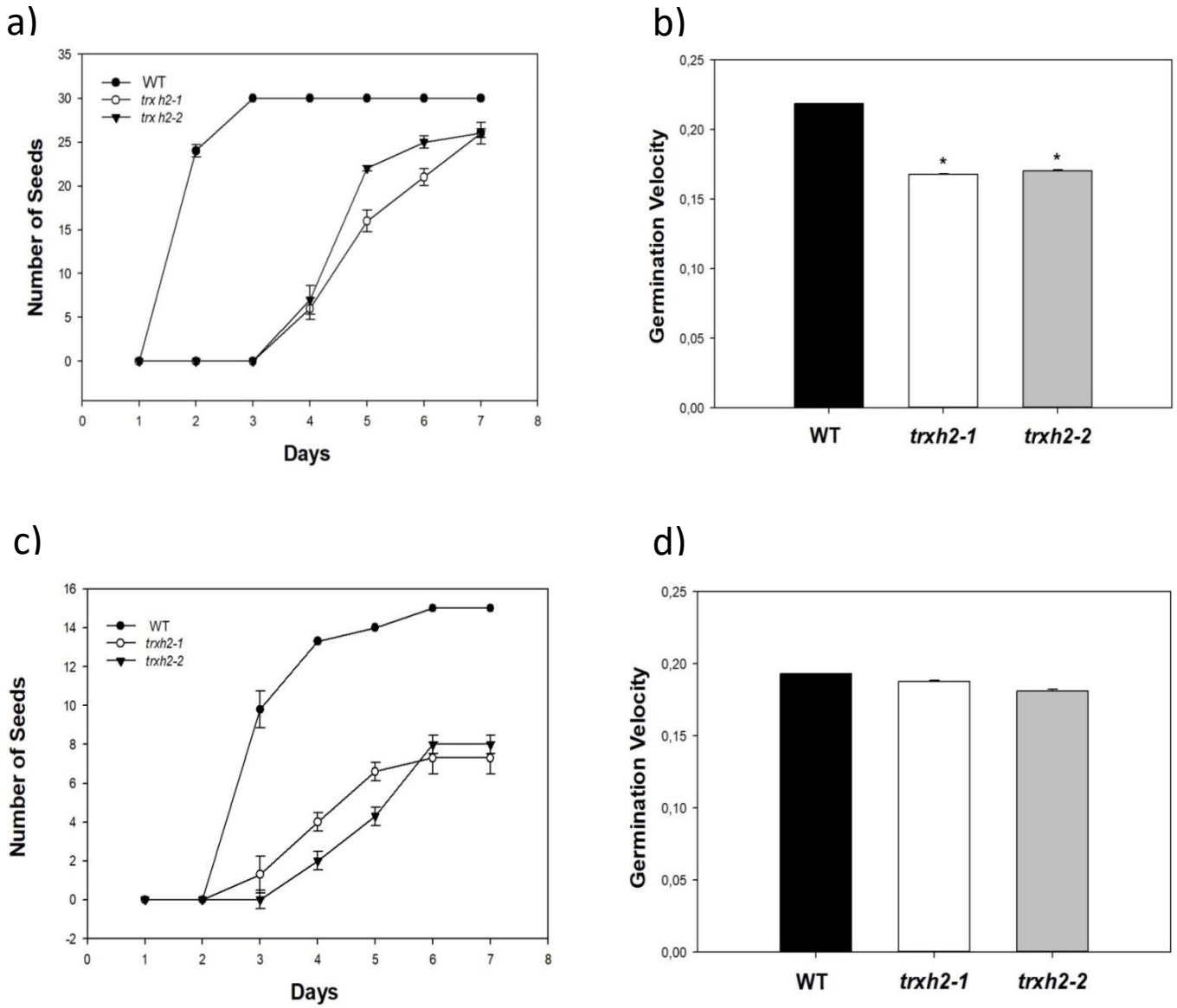


Figure 9 -Photochemical parameters a) ETR PSII b) ETR PSI curves in leaves oh Wild Type (WT) and Trx h2 Arabidopsis mutants, trxh2-1 and trxh2-2. The photosynthetic photon flux density (PPFD and temperature used in each measurement are described in the Materials and Methods section. Each point represents the average of four replicates.

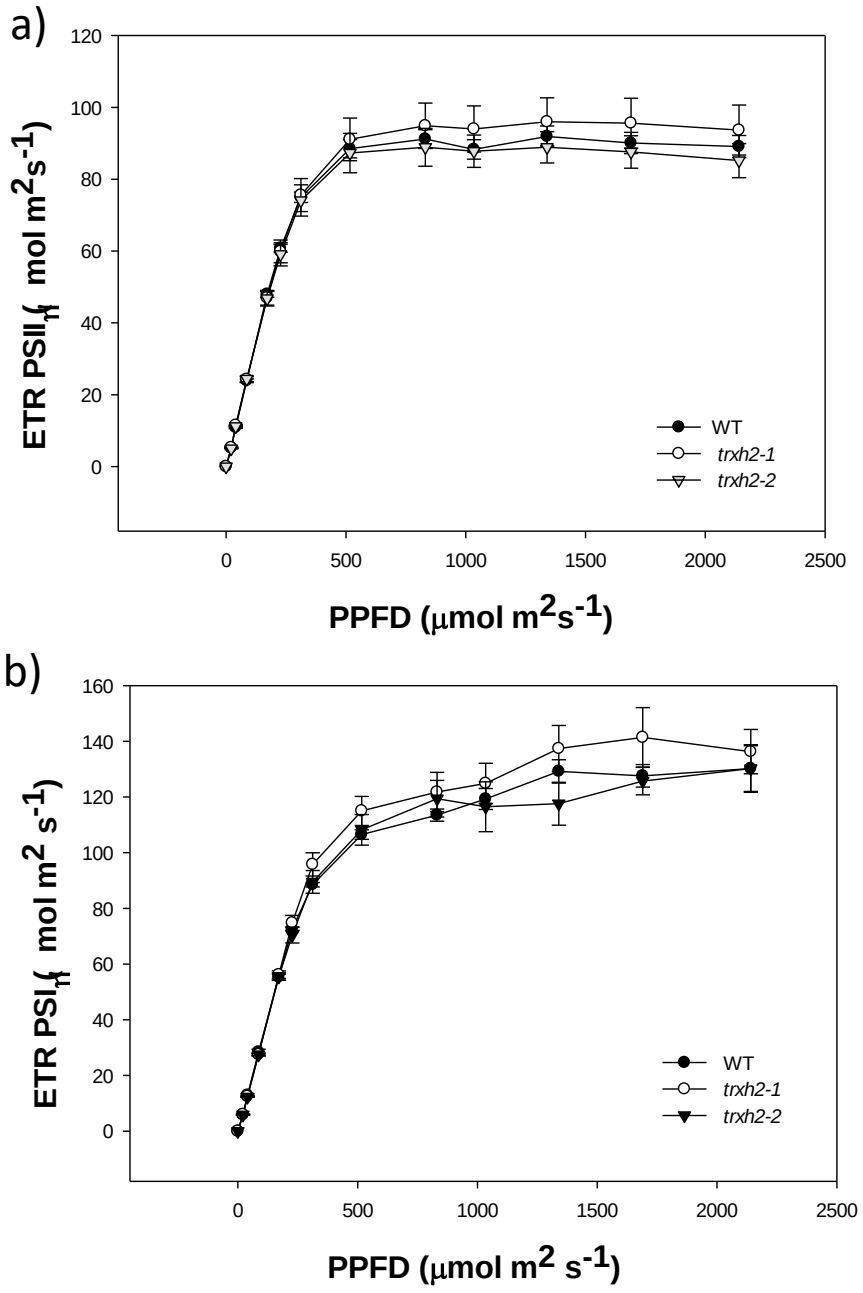
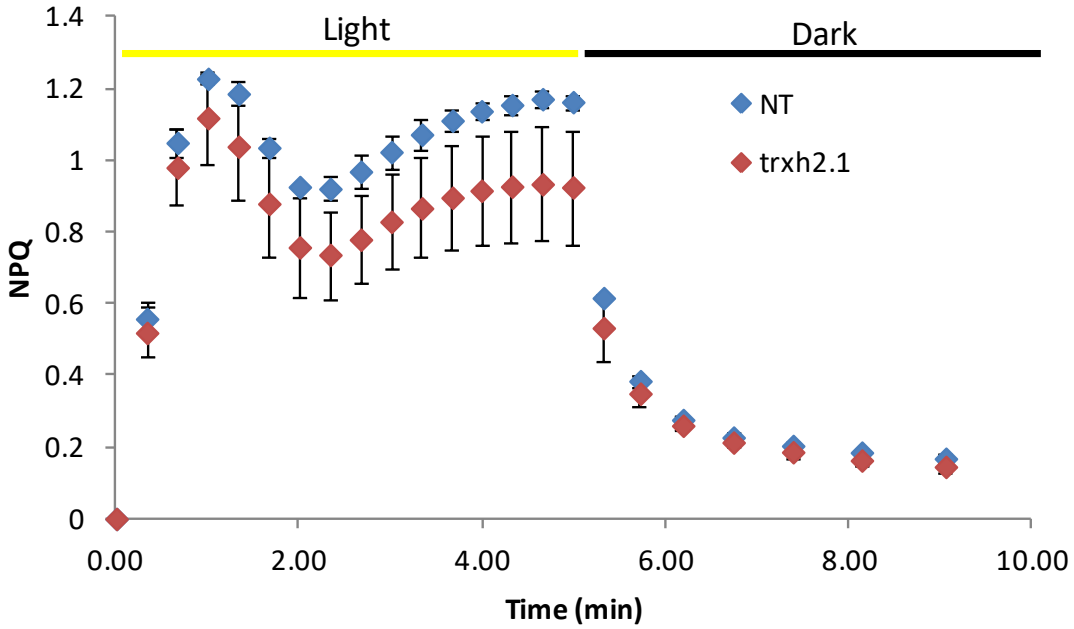


Figure 10- NPQ measure Kinetic in leaves from Non-Transformed and trxh2 (trxh2-1 and trxh2-2) deficient Arabidopsis thaliana. The actinic light employed in NPQ induction kinetics was 1000 µmol m^{-2} s^{-1}. The routine consisted of 5 min illumination followed by 5 min dark exposure relaxing NPQ values are average of 5 independent measures on different leaves (n=5). Error bars represent standard error (S.E.)

a)



b)

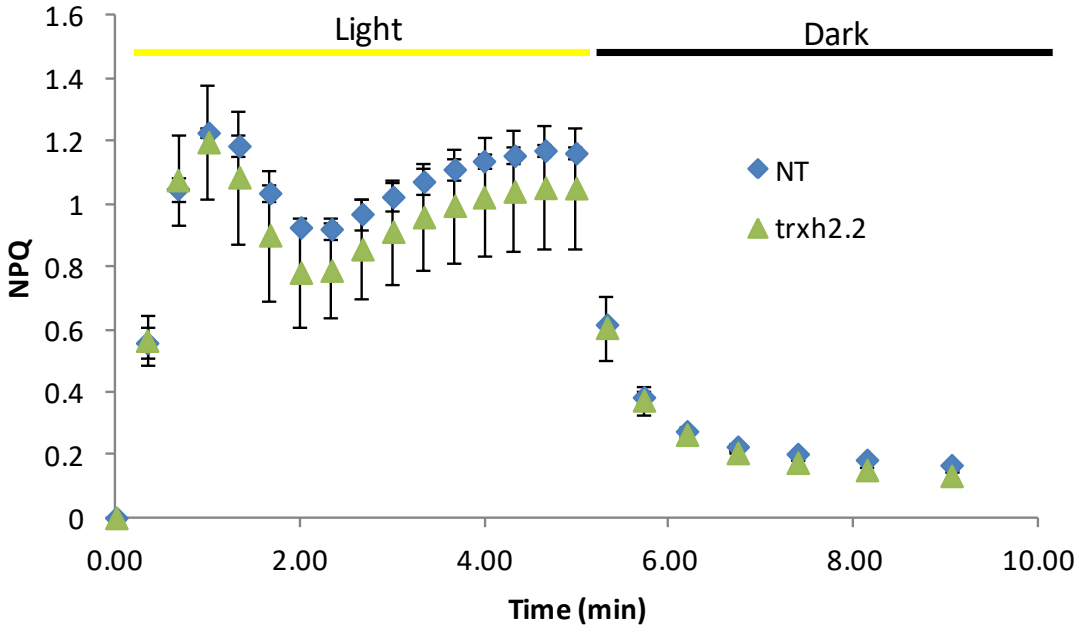


Figure 11- Gas exchange permeant, A/CI curve from Wild-Type, trxh2-1, and trxh2-2. The A/Ci curve was made with 4 replicates per genotype. Stomatal Conductance (g_s) and transpiration Rate were calculated from A/Ci curve, and no statistical significance changes were presented on trxh2 mutants in relation with WT. Each point represents the average of 4 repetition carried out 1000, 400, 300, 200, 100, 50, 400, 600, 800, 1200, 1500 μmol of CO_2.

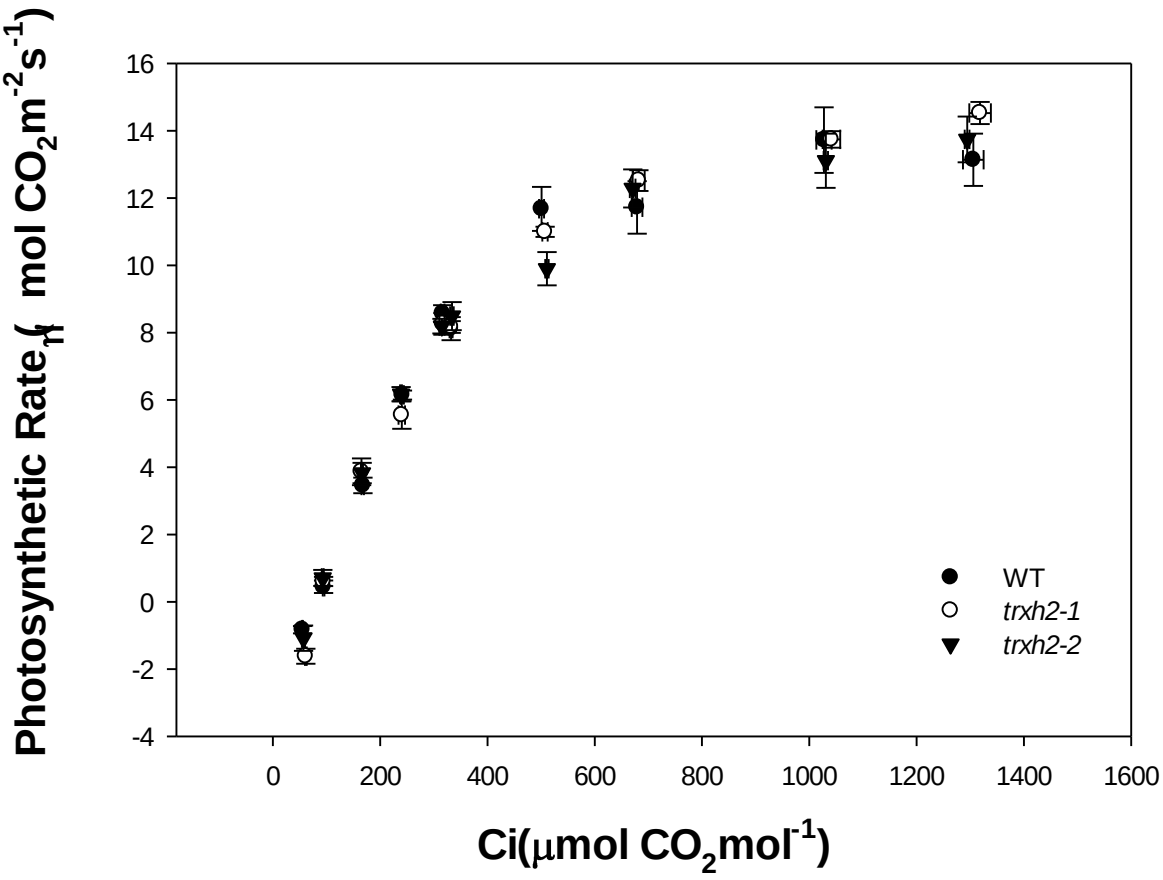


Figure 12 - Photorespiration rate from WT and trxh2 mutants. The photorespiration rate was calculated according (YOSHIMURA e colab., 2001). The numbers of independent replicates were 8 per genotype. Error bars represent standard error (S.E.).

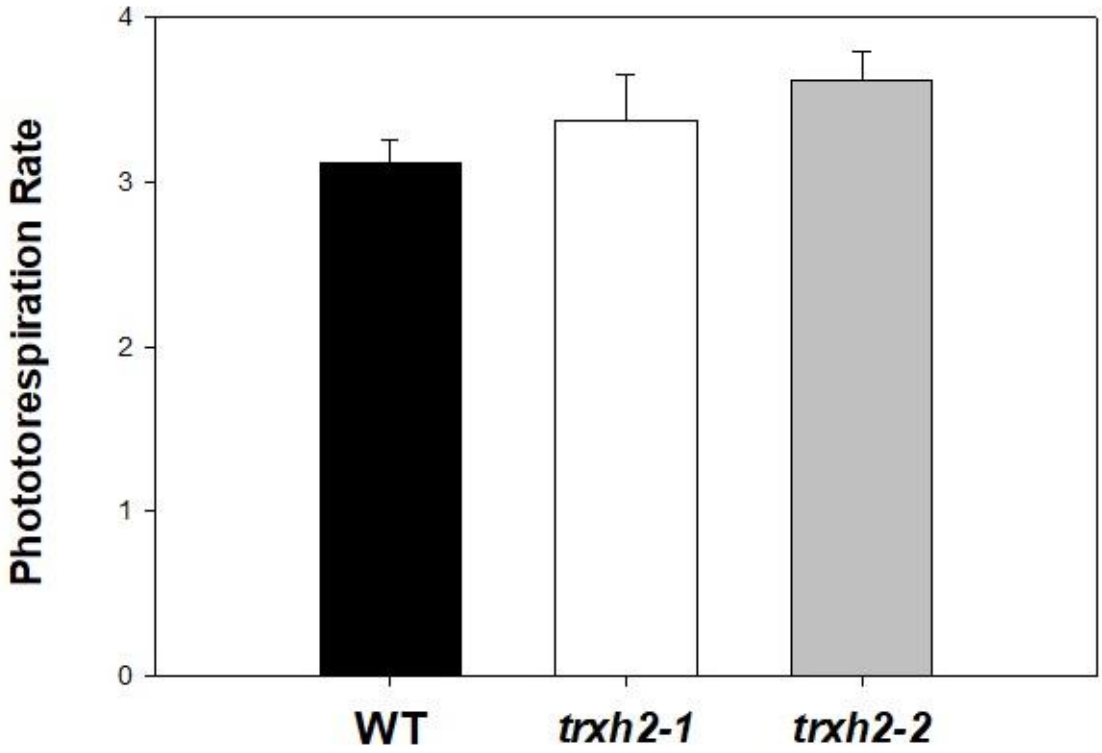


Figure 13 - Changes in nucleotides in levels of trxh2-1 and trxh2-2 mutants compared with Wild-Type (WT). Sum of a) NAD+, b) NADP+, c) NADH, d) NADPH, e) NADH/NAD+ ratio, f) NADPH/NADP+ ratio was measured in leaves harvested at the EN and ED. The numbers of biological replicates were 6 per genotype. The results were evaluated with T Student T-test P <0,05, statistical difference is represented by asterisk. Error bars represent standard error (S.E).

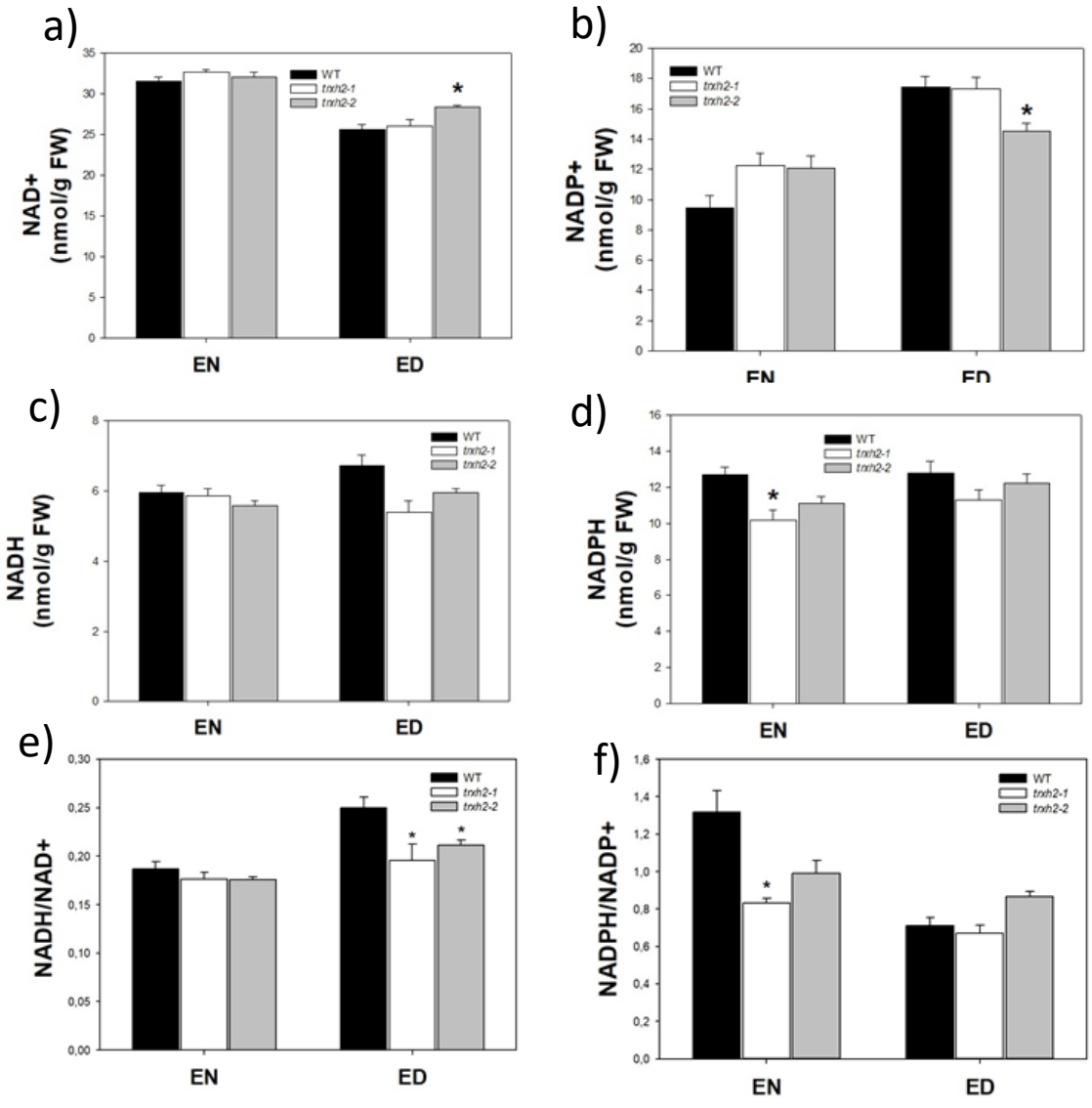


Figure 14 - Overview of metabolites profiles WT, trxh2-1, trxh2-2 in a systematic view. Here we provided all metabolites alteration in mutants deficient in Trx h2 and how metabolic pathway are linked. Dotted lines represent an indirect reaction, while arrow direct reaction. In addition, the results were evaluated with T Student T-test P <0,05, statistical difference are represented by asterisk. Error bars represent standard error (S.E).

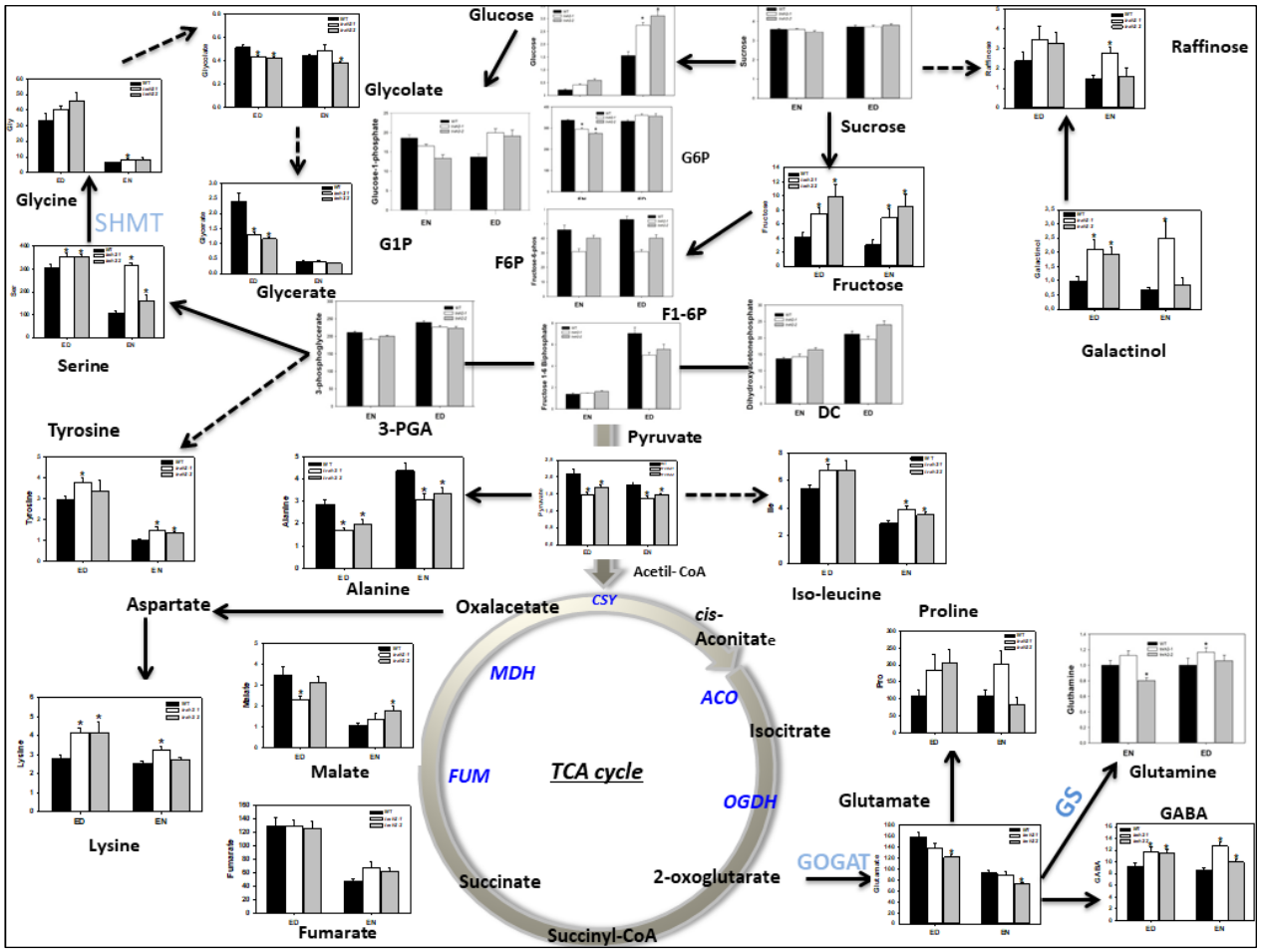


Figure 15 - The metabolite profile of: a) sucrose b) Glucose c) Starch d) Fructose-6-phosphate e) Glucose-6-phosphate f) Glucose-1-phosphate g) 3-phosphoglycerate, h) Dihydroxyacetone phosphate, and i) Fructose-1,6-biphosphate (nmol/g FW). Carbohydrate metabolites profile of trxh2 mutants compared with WT. The number of replicates for genotype was 6 (N=6). The asterisk represents statistical significance (T-Test Student P>0,05) difference mutants compared with WT. Error bars represent Standard Error (S.E).

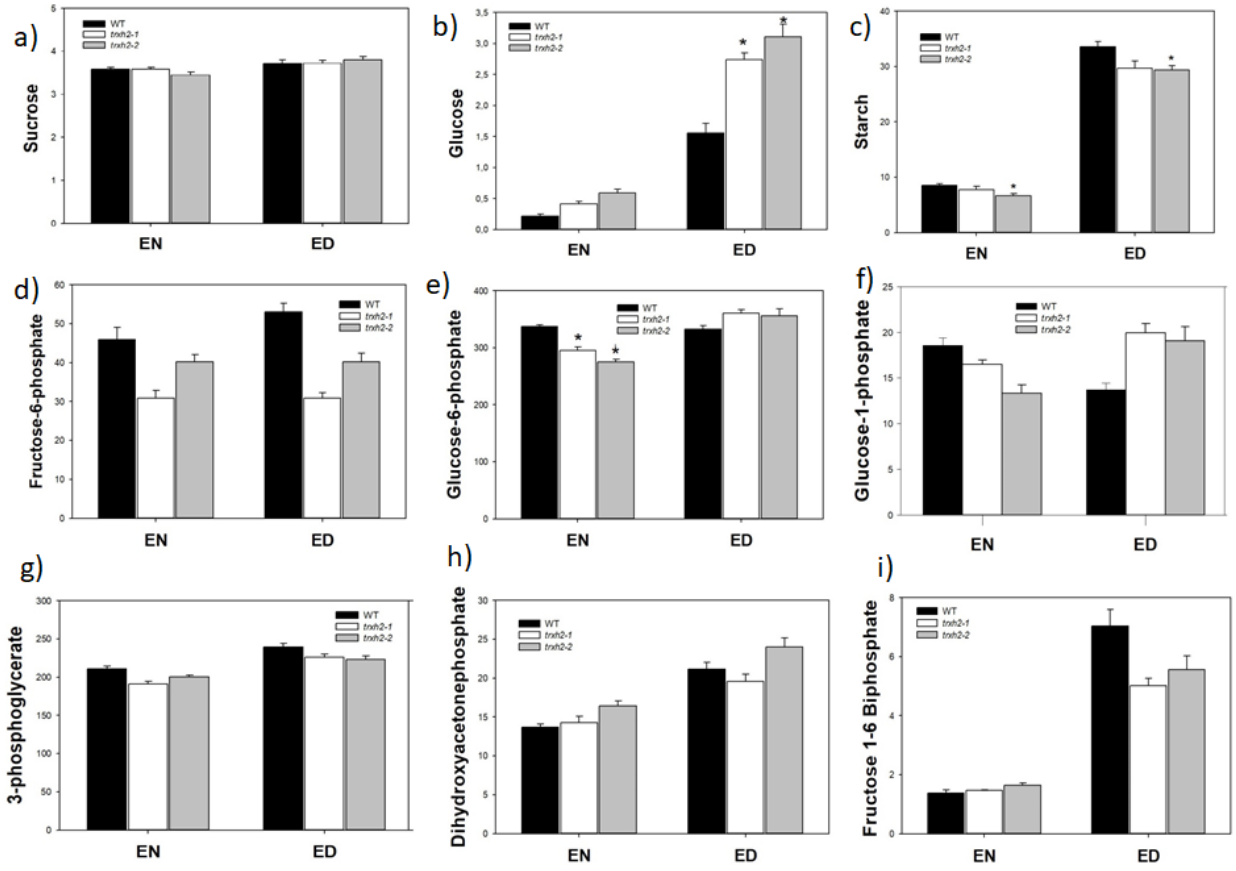


Figure 16 - Heat map representing the changes in relative abundance of metabolite profile in short-day-grown Arabidopsis knockout mutants trxh2-1, trxh2-2 and Columbia wild type plants (Col-0) harvested at two time points: end of the night (EN) and end of the day (ED) as measured by GC-MS. Relative log2-transformed values of signal intensities were normalized with respect to the mean response calculated for the wild type control at end of the night. Values are means ± SE of six independent samplings; asterisks demarcate values that were judged to be significantly different from the WT ($P < 0.05$) T-Student.

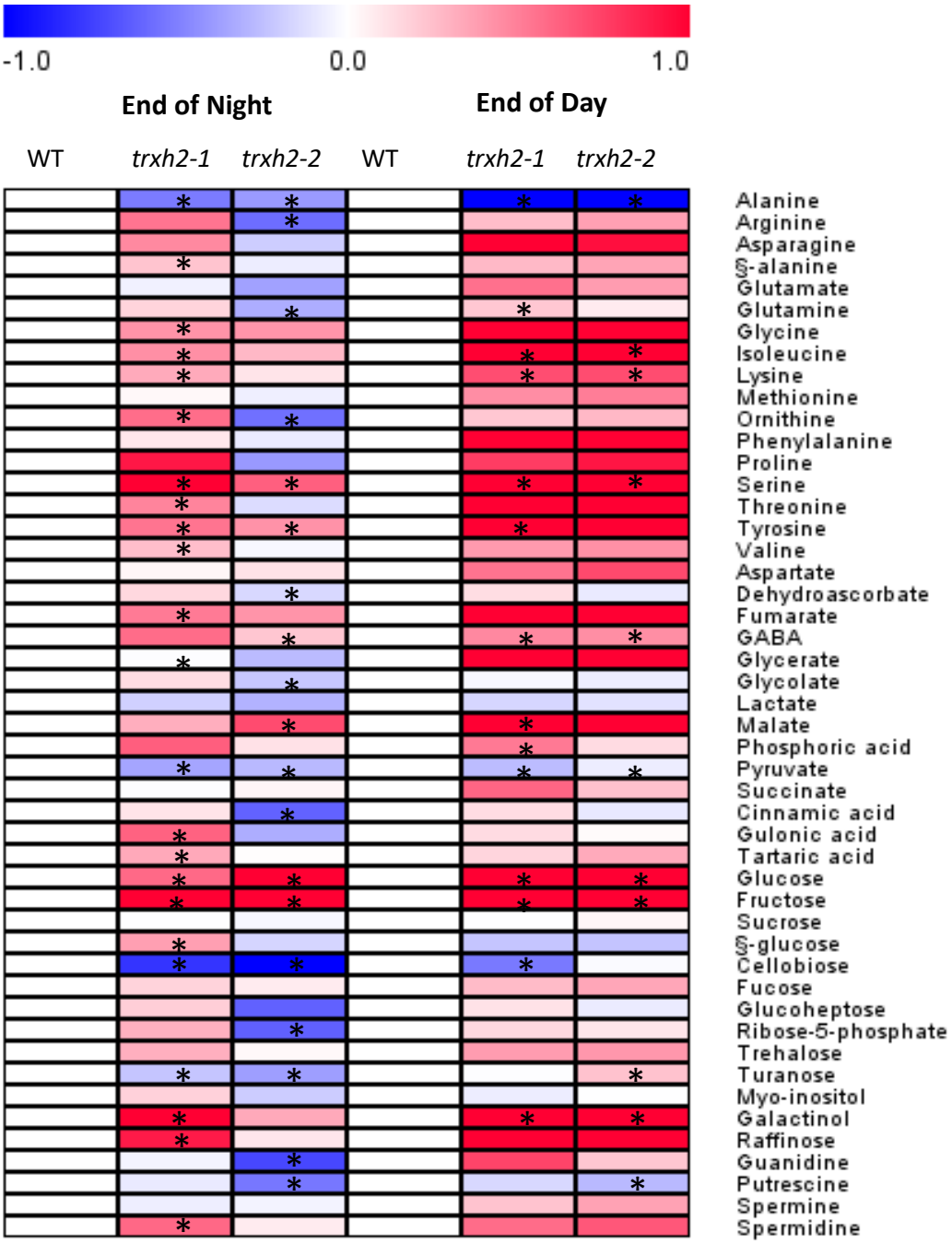


Table 4 - Dianna's program disulfide bridge prediction. The enzymes chosen are involved in photorespiration, TCA cycle, respiration, and Nitrogen assimilation. The gene number assertions are given in this table as well. The Diaana program gives a score according to (FERRÈ; CLOTE, 2005). Furthermore, the program Dianne gives the Cys residues on the protein sequence, and the amino acid distance between the possible disulfide bridges.

Enzymes	Tair	Cystein sequence position	Distance in aa	Score
Glycine Decarboxylase H	AT2G35370	33-47//43-70//45-57//45-70	14//2//12/25	0.995//0.998//0.995
Glycine decarboxylase P1	AT4G33010	98 - 245//228 - 280//253 - 463//402 - 777//569 - 768	147//28//61//199	All above 0.99
Glycine decarboxylase P2	AT2G26080	103 - 251//234 - 286//259 - 670//408 - 783//575 - 774	10//11//14	All above 0.99
Glycine decarboxylase T	AT1G11860	221 – 276	55	0.991
Serine Hidroxymethyl Transferase	AT4G37930	-----	-----	Lower than 0.03
Alanine Glyoxylate Aminotransferase	AT2G13360	66-382	----	0.678
Hydroxypyruvate reductase 1	AT1G68010	----	---	Lower than 0.02
Glutamine 2-oxoglutarete aminotransferase	AT5G53460.1	132-168//132-1490//282-479	34//1368//197	0.987//0986//0.988
Glutamine Syntheses 2	AT5G35630	101-168	67	0.112
Glycolate Oxidase	AT3G14420	100-114	14	0.999
Phosphoglycolate Phosphatase	AT5G36700	----	----	Lower than 0.02
Cyt and Mit Fumarase (FUM 2)	AT5G50950	302-415//302-461	113//159	0.991//0998
Mit Fumarase (FUM 1)	AT2G47510	----	-----	Lower than 0.02
Citrate synthase	AT2G11270	-----	-----	Lower than 0.02
Succinate Dehydrogenase	AT1G08480	179 - 261//261 – 433	41//82//172	All presented score 0.997
Isocitrate Dehydrogenase	AT3G09810.1	275 – 371	96	0.667
Aconitase	AT4G35830	338-368//338-407//338-766	30//69//428	0.999//0.999/0.999
Mitochondrial Malate Dehydrogenase	AT1G53240	----	----	Lower than 0.02
Cytosolic Malate Dehydrogenase	AT1G04410	----	----	Lower than 0.02
Alternative Oxidase	AT3G22370	----	----	Lower than 0.02

Figure 17 - Dimension protein structure models view. The 3D protein models of a) GDC-P b) SHMT c) GDC-T d) GDC-P2. The protein models were built with Arabidopsis thaliana desire protein sequence input and create in relation with a protein Template from Protein Data Bank (PDB). All protein templates and the parameters for model 3D validation are at table II. Represent by sphere the disulfide bridge automatically create in the model, in yellow predict disulfide bridge (GDC-T) through Dianna predict scores. All others protein models are at supplement.

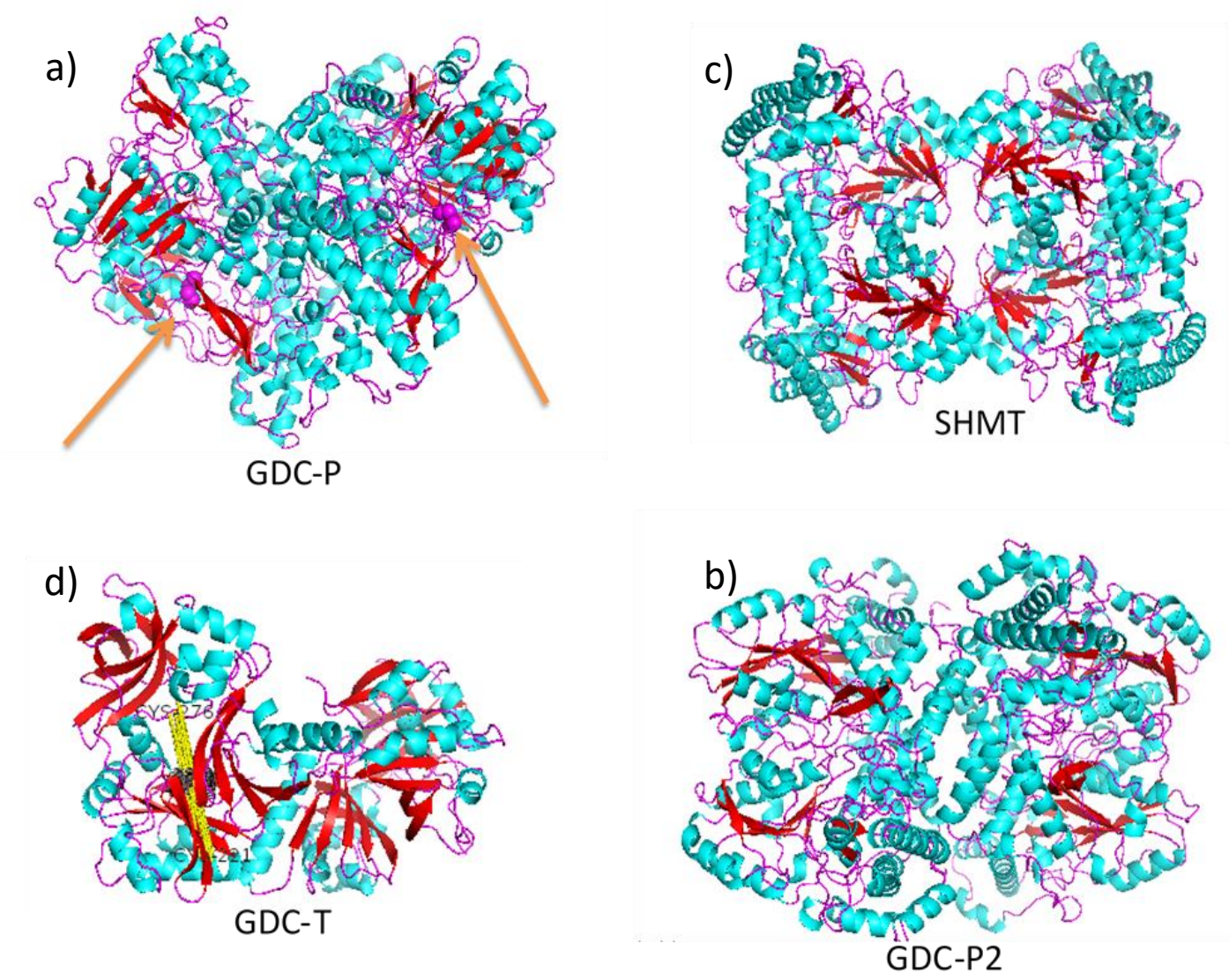


Table I - The protein models parameters validation. In the table are all parameters validations describe at material and methods section. In addition, all protein templates from PDB are at this table. All protein validations were evaluated according to(BIASINI e colab., 2014; YEATS e colab., 2006).

Enzymes	Tair	Similarity sequence	Qmean	GMQE	Template From PDB
Glycine Decarboxylase H	AT2G35370	----	-----	-----	-----
Glycine decarboxylase P1	AT4G33010	0.5907	0.76	-1.07	4lgl.1.A
Glycine decarboxylase P2	AT2G26080	0.5907	0.76	-1.07	4lgl.1.A
Glycine decarboxylase T	AT1G11860	0.5903	0.77	-1.16	4lgl.1.A
Serine Hidroxymethyl Transferase	AT4G37930	0.6256	0.77	0	4pvf.1.A
Alanine glyoxylate aminotransferase	AT2G13360	----	-----	-----	-----
Hydroxypyruvate reductase 1	AT1G68010	0.3849	0.62	-2.83	5aov.1.A
Gluthamate 2-oxoglutarate aminotransferase	AT5G53460	----	-----	-----	-----
Glutamine Synthetize 2	AT5G35630	----	-----	-----	-----
Glycolate Oxidase	AT3G14420	0.9029	0.92	0.47	1al7.1.A
Phosphoglycolate phosphatase	AT5G36700	0.5188	0.73	0.09	1wsv.1.A
Fumarase (FUM 2)	AT5G50950	0.7153	0.77	-0.63	3e04.1.D
Mitochondrial Fumarase (FUM 1)	AT2G47510	0.7152	0.82	-0.3	5upp.1.A
Citrate synthase	AT2G11270	0.6256	0.79	-1.04	1al6.1.A
Succinate Dehydrogenase	AT1G08480	0.7148	0.8	-1.16	4ytp.1.A
Iso citrate Dehydrogenase	AT3G09810	0.3372	0.69	-1.06	1hqs.1.A
Aconitase	AT4G35830	0.6139	0.82	-0.05	2b3y.1.A
Mitochondrial Malate Dehydrogenase	AT1G53240	0.644	0.78	-0.51	1sev.1.A
Cytosolic Malate Dehydrogenase	AT1G04410	0.9398	0.98	1.13	5nuf.1.A
Alternative Oxidase	AT3G22370	0.4181	0.52	-4.3	3w54.2.A

Figure 18 - Immunoblot detection of SHMT in WT, trxh2-1, and trxh2-2. Here is representing the Western blot of SHMT (using anti-SHMT, dilution 1:1000) with composite samples compost for 3 independent biological replicates (n=3) per genotype. To quantify was made a WT gradient representing per 50%, 75 %, and 100% of total protein WT, being added 25 ug protein for genotype (corresponding 100% WT) per well acrylamide gel 12,5%. Furthermore, was made three blot immune blots replicates. In addition, was made T-student test, being representing by asterisk the statistical difference. Error bar represents the standard error (S.E).

50 %WT 75% WT 100% WT *trxh2-1* *trxh2-2*

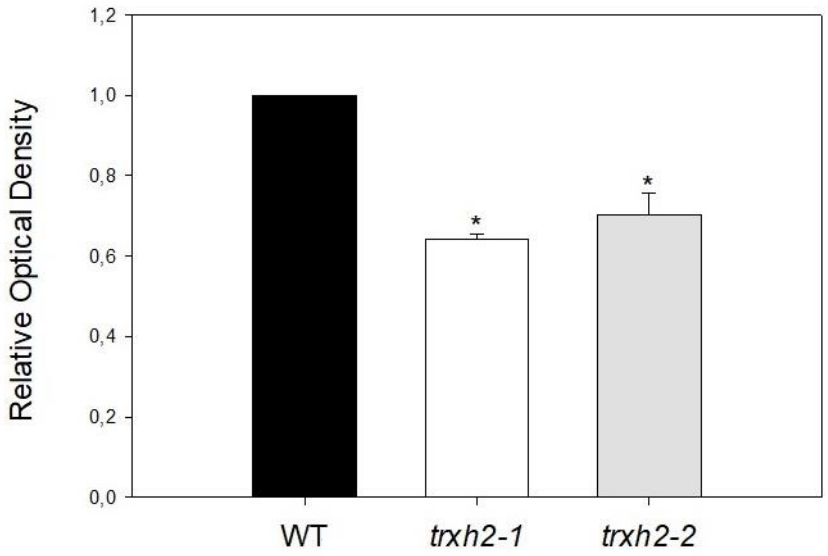
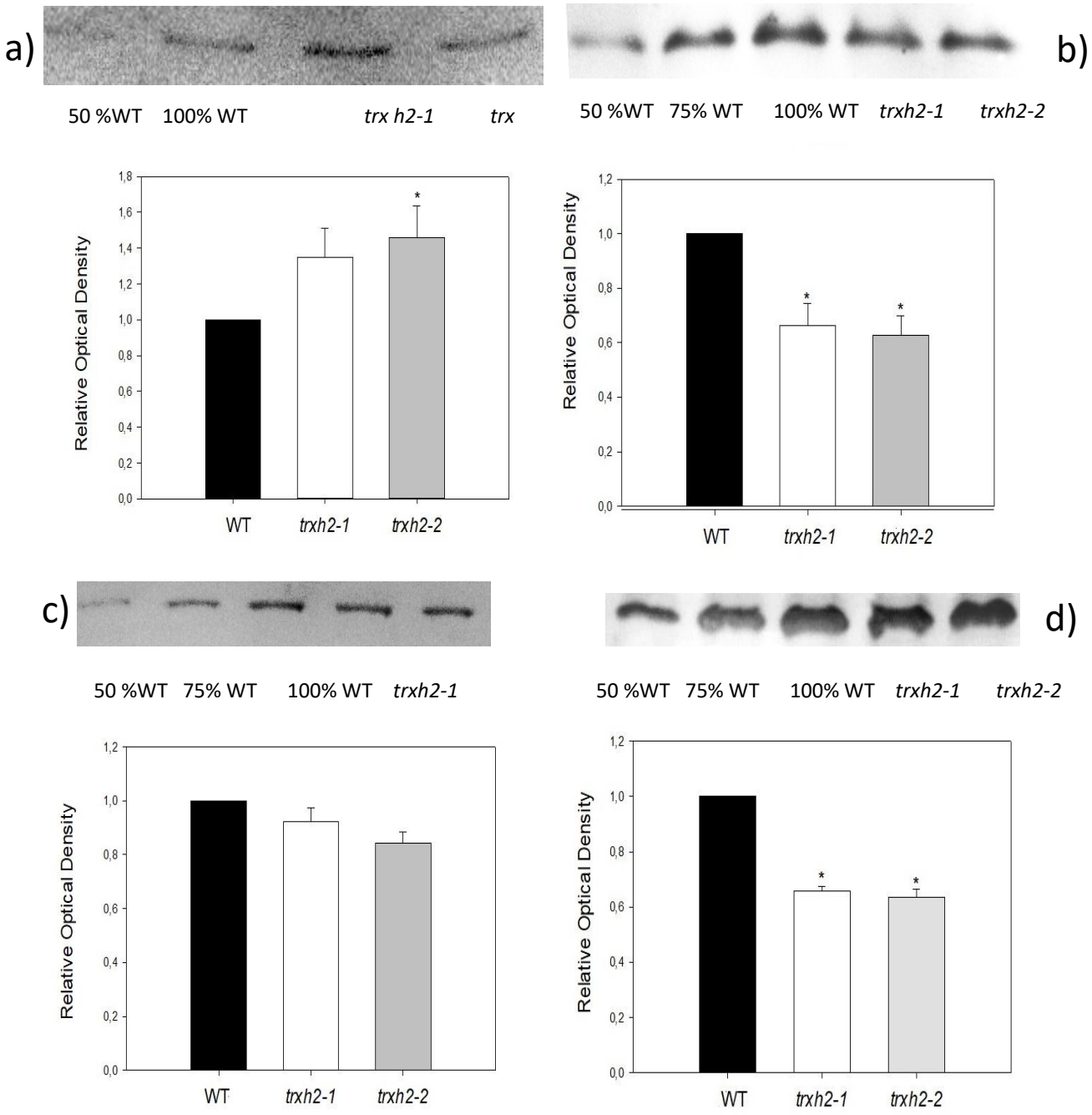


Figure 19 - Immune detection of complex Glycine Decarboxylase in WT, trxh2-1, and trxh2-2. Here is representing the Western blot of GDC complex (using anti-GDCs, GDC-P 1:2000, GDC-L 1:2000, GDC-H 1:3000, GDC-T 1:2000) with composite samples compost for 3 independent biological replicates (n=3) per genotype. To quantify was made a WT gradient representing per 50%, 75 %, and 100% of total protein WT, being added 25 ug protein for genotype (corresponding 100% WT) per well acrylamide gel 12,5%. Furthermore, was made three immune blots replicates for each protein in analyze. In addition, was made T-student test, being representing by asterisk the statistical difference. Error bar represents the standard error (S.E).



APPENDIX B - SUPPLEMENT

Figure 20 - Arabidopsis thaliana Trx h2 gene structure (A) Boxes indicate exons and lines indicate introns. T-DNA insertions were identified in the second intron and in the third exon of trxh2-1 and trxh2-2, respectively. Relative expression levels by RT-PCR using the housekeeping elongation factor EF1a gene as an internal control (B) and real time quantitative PCR (qPCR) (C); the relative quantification of Trx h2 expression was made using GAPDH and β-actin as housekeeping genes.

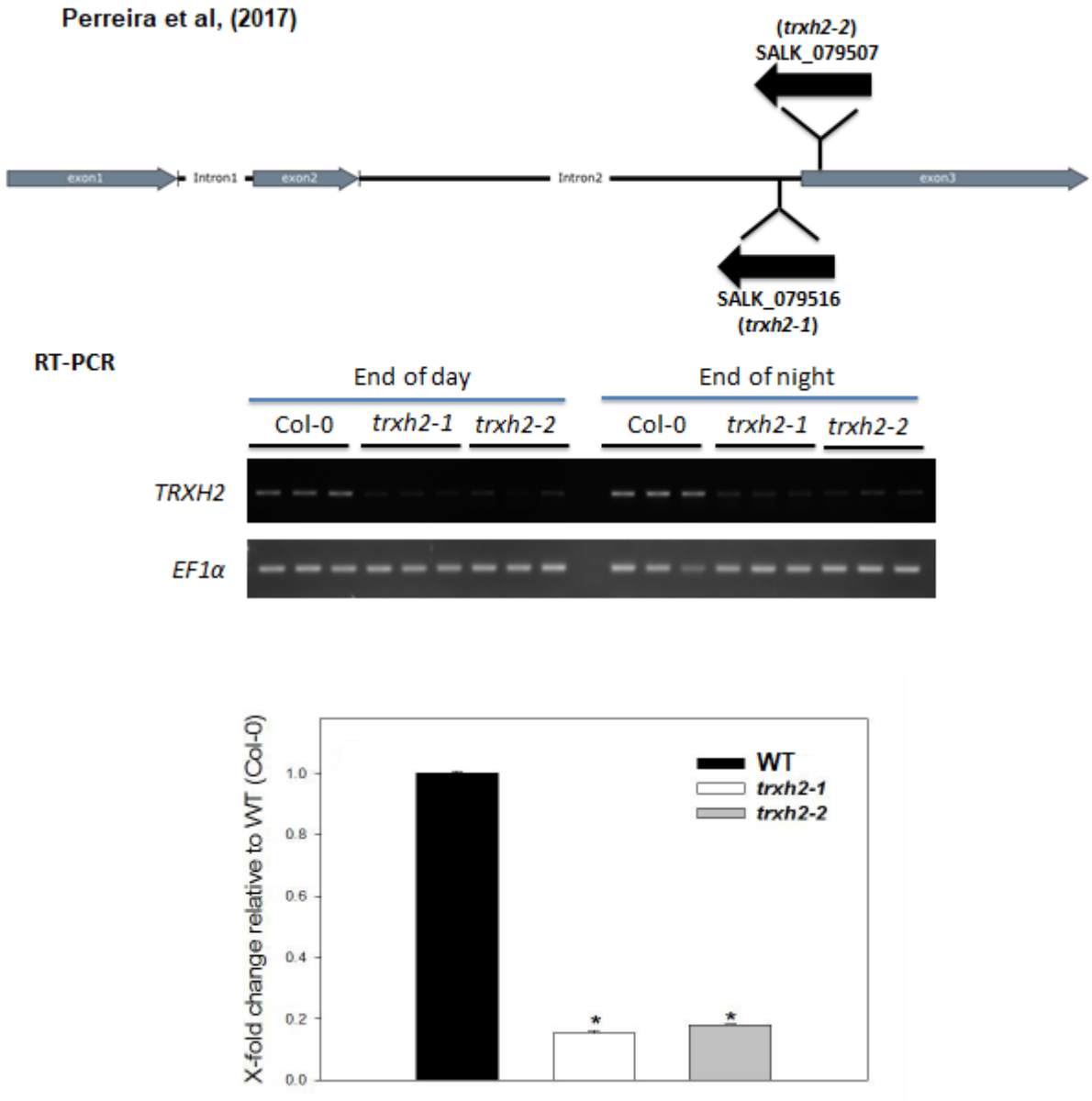


Figure 21 - Photochemical results Fv/Fm of WT, trxh2-1, and trxh2-2. The Fv/Fm was measured in the Dark. The plants grew in 120 μmol photon m^{-2} s^{-1}, 55 % relative humidly, and short-day photoperiod (9 light/ 15 dark).

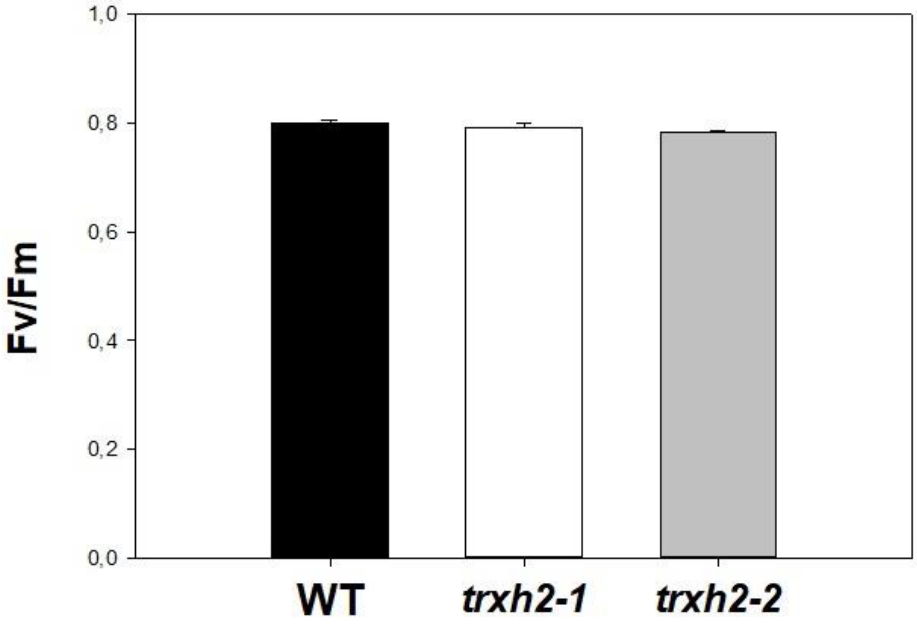


Figure 22 - In silico analyze Arabidopsis thaliana Trx h2 transcripts level along of Arabidopsis development. The Transcript shoot abundance is representing by yellow-orange gradient.

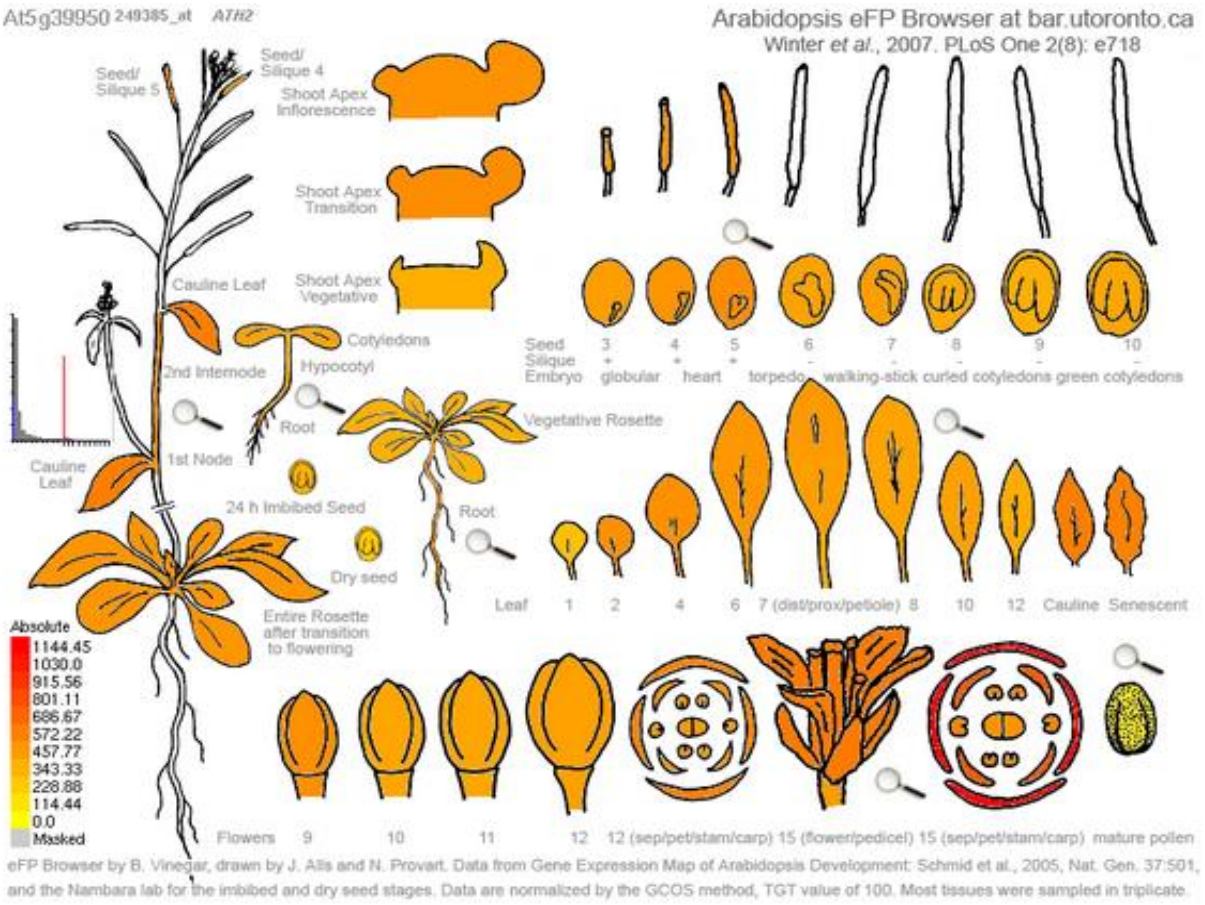


Figure 23 - Interactomic generated from Cytoscape version 3.60. In addition, each rectangular represent a protein, while each node (representing by line) represent an interaction protein. The gene assertion numbers for creation of this interactomic were from Arabidopsis thaliana, Trx h2 (AT5G39950). Here, the protein-protein interaction between Trx h2 on the center. Marked in yellow, all proteins which interact with Trx h2: Methione Sulforedoxin Reductase (MSR), Cytoplasmatic Thiol Peroxidase (TSA1), CDC33 a DNA-binding protein important for protein biosynthesis, and Thioredoxin disulfide reductase of yeast (TRR1).

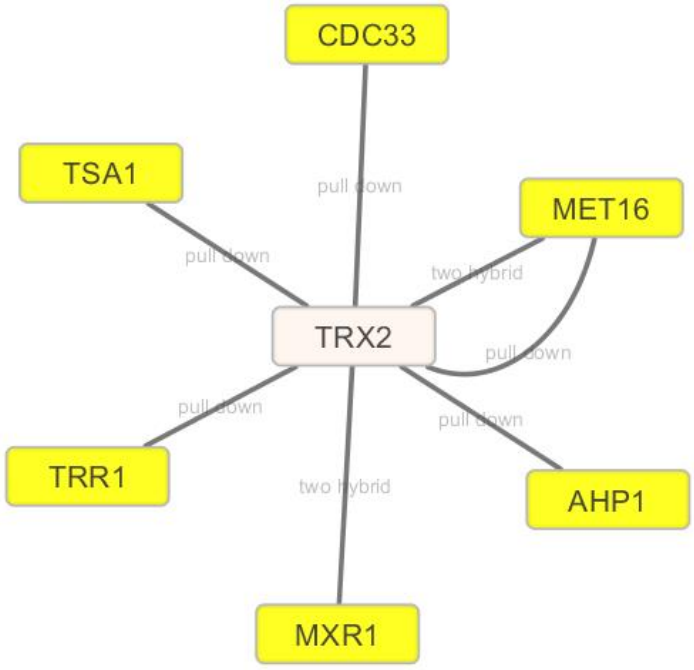


Figure 24 - GDC-L clustaw omega alignment among Arabidopsis thaliana, Camelina Sativa, Brassica rapa, Capsella rubella, Raphanus sativus, Cucumis melo, and Morus notabilis. Here is represent the partial alignment of GDC-L. Marked in green the conserved Cysteine residue, in black shade the conserved site between all sequence input, and grey shade marked conserved residues in almost sequences input. All others protein alignments are at supplement. In addition, all protein sequence is at supplement.

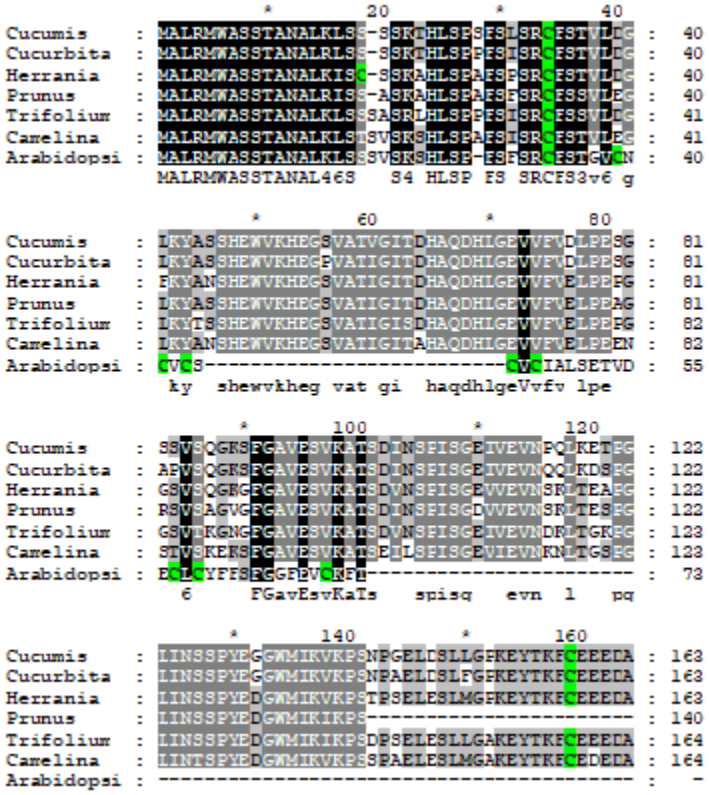


Figure 25 - Co expression Trx h2 by String database (SZKLARCZYK e colab., 2017). Trx h2 correlation among NADPH Thioredoxin Reductase A and B, Glutamine Synthases 1 and 2, Peroxiredoxins Q and IIF(PRXIIF), and Glutamate transport 1 (GLT-1). Each color line represents a kind of correlation, purple gene experiments correlated, blue lines represent gene correlation based in databases, green lines is genes neighborhood, in black genes that are co-expressed.