UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE BIOCIÊNCIAS

PROGRAMA DE PÓS-GRADUAÇÂO EM GENÉTICA E BIOLOGIA MOLECULAR

CARACTERIZAÇÃO FUNCIONAL DO GENE *OsGPX3* QUE CODIFICA UMA GLUTATIONA PEROXIDASE MITOCONDRIAL EM ARROZ

Tese de Doutorado

ANA LUIZA SOBRAL PAIVA

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Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul, como requisito para a obtenção do grau de Doutor em Ciências (Genética e Biologia Molecular)

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RESUMO

O arroz (*Oryza sativa*) é uma das espécies mais importantes do mundo e um excelente modelo para entender a interação entre genes e as mudanças ambientais. Entretanto, sua produtividade é comumente desafiada por muitos estresses, como os abióticos e oxidativos. As glutationa peroxidases (GPXs) fazem parte do mecanismo pelo qual as plantas lidam com o estresse oxidativo. GPXs podem controlar a equilíbrio redox atuando também na sinalização celular. Neste trabalho investigou-se o papel do gene *GPX3* de arroz em resposta ao estresse salino, usando plantas RNAi com o gene *OsGPX3* silenciado (GPX3s). Os resultados indicam que essas plantas são mais sensíveis à salinidade, mostrando menor biomassa, assimilação de CO_2, condutância estomática e pressão parcial intercelular de CO_2. Plantas GPX3s também apresentaram significantes danos na atividade do fotossistema II e declínio no conteúdo de clorofila. O estresse salino induziu acúmulo de espécies reativas de oxigênio (ERO) em ambas plantas, NT (não transformadas) e GPX3s, indicando que a sensibilidade das plantas GPX3s ao sal não é devida à uma significante deficiência no equilíbrio redox. Para elucidar as rotas reguladas por *OsGPX3*, utilizou-se a técnica de proteômica livre de marcação, comparando plantas NT e GPX3s. Plantas GPX3s apresentaram alterações na abundância de proteínas relacionadas com resposta ao ABA e processos epigenéticos. RT-qPCR e coloração usando linhagens repórter mostraram que o gene *OsGPX3* é induzido pelo tratamento com ácido abscísico (ABA), sugerindo que esse gene pode desempenhar um importante papel na via de sinalização do ABA. A aplicação exógena de ABA não inibiu a germinação das sementes, tampouco induziu acumulação de ERO e o fechamento estomático em GPX3s. Ademais, GPX3s e NT apresentaram fenótipo similar ao de plantas submetidas ao estresse de seca. Entretanto, GPX3s foram mais sensíveis à indução da senescência no escuro promovida por ABA. Esse trabalho fornece importantes informações sobre a interrelação entre cloroplastos e mitocôndrias, mostrando a importância da proteína GPX3 na aquisição da tolerância à salinidade em arroz via mecanismos independente da acumulação de ERO. Além disso, é um estudo pioneiro demonstrando o papel do *OsGPX3* na sinalização do ABA, corroborando com o

fato de enzimas antioxidantes agirem em diferentes e complexas vias nas células.

Palavras-Chave: *Oryza sativa*; Mitocôndria; Salinidade; Ácido Abiscísico

ABSTRACT

Rice is one of the world's most important crops and an excellent model system for understanding the interaction between genes and environmental changes. However, its productivity is often challenged by stresses, as abiotic and oxidative. Glutathione peroxidases (GPXs) are part of the mechanism by which plants cope with oxidative stress. GPXs can control redox homeostasis and also play a role in redox signaling. Here, we investigated the rice GPX3 role in plant responses to salt stress using *OsGPX3*-RNAi silenced rice plants (GPX3s). Results indicate that GPX3s plants are more sensitive to salinity showing decreased biomass, CO_2 assimilation rate, stomatal conductance, and intercellular CO_2 partial pressure. These plants also present significant damage to photosystem II activity and decline in chlorophyll content. Salt stress induced ROS accumulation in both non-transformed (NT) and GPX3s plants, indicating that GPX3s sensibility to salt stress was not due to the significant impairment in redox equilibrium. To elucidate the routes regulated by *OsGPX3* we performed a proteomic approach comparing NT and GPX3s. The GPX3s plants presented altered the abundance of proteins involved in abscisic acid (ABA) response and epigenetic processes. RT-qPCR and GUS-staining using reporter gene lines to GPX3 promoter showed that *OsGPX3* is induced by ABA treatment, suggesting that this gene could be important in ABA pathway. The analysis of ABA-related responses showed that ABA is unable to inhibit seed germination, ROS accumulation and stomata closure in GPX3s plants. GPX3s and NT plants presented similar phenotype under drought stress. However, GPX3s were more sensitive to dark-induced senescence after ABA treatment compared with NT plants. Together, this work provides new light into the cross-talk between chloroplasts and mitochondria, showing GPX3 protein importance in rice to achieve salt stress tolerance via an ROS-accumulation independent mechanism, not acting as the major ROS-scavenger enzyme. Moreover, it also suggests a novel role to this enzyme beyond its role as ROS-scavenger, as a signaling compound. This is a pioneer study demonstrating that *OsGPX3* play a role in ABA signaling and corroborate that redox homeostasis enzymes can act in different and complex pathways in plants cells.

Keywords: *Oryza sativa*; Mitochondria; Salinity; Abscisic acid

1. INTRODUÇÃO

A genômica funcional é um campo da biologia molecular que descreve a função e interação de genes e proteínas por meio de abordagens metodológicas aplicadas ao genoma (BUNNIK; LE ROCH, 2013). Essas abordagens combinam dados provenientes de vários processos, como de sequenciamento de DNA, expressão gênica, transcrição de RNA codificante e não-codificante, tradução de proteínas, interação entre as moléculas (proteína-DNA, proteína-RNA e proteína-proteína), função proteica, entre outros. Esses dados são usados de forma integrada para desenvolver modelos interativos e dinâmicos que ajudam a elucidar o funcionamento de diferentes processos dos organismos. A genômica funcional pode ser aplicada ao estudo de diversos processos biológicos tais como origem e mecanismos de doenças, ciclo celular, desenvolvimento dos organismos, resposta a estresses, entre outros; assim como para aplicações biotecnológicas, como na prospecção de biomoléculas.

Na biologia vegetal, a maioria dos estudos é iniciado com uso de plantas como *Arabidopsis thaliana* e arroz (*Oryza sativa*), que representam plantas-modelo no grupo das dicotiledôneas e monocotiledôneas, respectivamente. O uso dessas plantas tem inúmeras vantagens, mas uma das principais é o fato de haver protocolos e técnicas moleculares bem estabelecidas para a manipulação desses organismos, o que facilita na aplicação de diferentes abordagens metodológicas.

Uma das aplicações da genômica funcional em plantas modelo mais usadas é busca de genes envolvidos nas resposta a estresse. As plantas estão continuamente expostas a condições ambientais desfavoráveis e, por serem sésseis, precisam de eficientes mecanismos bioquímicos, morfológicos e fisiológicos para sobreviverem. Por meio de sistemas sensores, elas são capazes de perceber o estresse, seja ele biótico ou abiótico, levando à ativação de complexas redes regulatórias e de sinalização, controlando a expressão de genes efetores para combater os efeitos adversos e para tentar estabelecer a homeostase celular. Assim, o uso de abordagens “ômicas” aplicadas a resposta de plantas a estresses é importante para o entendimento de como as respostas

de sinalização são integradas e pode contribuir, por exemplo, para o desenvolvimento de cultivares mais resistentes e adaptadas ao meio ambiente.

O estresse oxidativo é o resultado do acúmulo de espécies reativas de oxigênio (ERO) e é desencadeado quando a planta está sob diferentes estresses. Para se proteger contra os danos da elevada concentração desses compostos, as plantas possuem sistemas enzimáticos e não-enzimáticos de eliminação das ERO. Entre esses sistemas, um é representado pela classe das enzimas glutationas peroxidases, as quais catalisam a redução de H_2O_2 e outros hidroperóxidos orgânicos a H_2O (BRIGELIUS-FLOHE; FLOHE, 2011).

Por muitos anos o estresse oxidativo foi estudado com foco apenas no equilíbrio entre ERO e o sistema antioxidativo das plantas. Acreditava-se que elevadas concentrações de ERO eram sempre prejudiciais às plantas e que as principais funções das enzimas antioxidativas era a remoção dessas moléculas. Entretanto, nos últimos anos, tem sido demonstrado que essas enzimas podem desempenhar funções em cascatas de sinalização, atuando como reguladoras do *status* redox celular ou interagindo diretamente com moléculas sinalizadoras como hormônios, revelando uma enorme complexidade apresentada por esses sistemas (FOYER; RUBAN; NOCTOR, 2017; PASSAIA; MARGIS-PINHEIRO, 2015).

O grupo de pesquisa liderado pela Profa. Márcia Pinheiro Margis vem se dedicando à caracterização funcional de genes que codificam GPX em arroz, utilizando abordagens de genética reversa. Foram identificados cinco genes codificando GPX no genoma de arroz através de análises *in silico* (MARGIS et al., 2008). A localização subcelular das diferentes isoformas codificadas por esses genes foi experimentalmente confirmada. Essas análises demonstraram que existem dois genes que codificam proteínas mitocondriais (*OsGPX1* e *OsGPX3*), uma citosólica (*OsGPX2*), uma cloroplastídica (*OsGPX4*) e uma citosólica ancorada ao retículo endoplasmático (*OsGPX5*). Esses genes são induzidos na parte aérea pela aplicação exógena de H_2O_2 e estresse por baixas temperaturas e reprimidos quando a planta é submetida a condições de seca ou tratada com luz UV-B. O silenciamento por RNAi do gene *OsGPX3* resultou em plantas jovens com raízes mais curtas e maior acúmulo de H_2O_2 quando comparadas às plantas não-transformadas. Esses resultados sugerem que o silenciamento dessa GPX mitocondrial afeta o desenvolvimento da planta jovem

e induz modificações morfológicas em resposta ao acúmulo do H_2O_2 (PASSAIA et al., 2013).

Apesar desses trabalhos terem contribuído para a caracterização das GPXs de arroz, ainda existem muitas lacunas e questões a serem elucidadas, especialmente com relação aos mecanismos envolvidos nas alterações fenotípicas e de resposta a estresse dependentes de GPX. A presente tese investiga a hipótese da participação da enzima GPX3 nas rotas de sinalização envolvidas no desenvolvimento do arroz, assim como nas suas respostas de defesa. Essas vias podem ser identificadas e estudadas utilizando a ferramentas de proteômica, comparando as proteínas diferencialmente acumuladas entre as plantas silenciadas para o gene *OsGPX3* e plantas não transformadas.

Esta tese está organizada em dois capítulos independentes e complementares, além de uma revisão bibliográfica e uma sessão final, apresentando uma discussão geral dos resultados. O primeiro capítulo descreve o papel do gene *OsGPX3* nas respostas de defesa contra estresses abióticos, com foco no estresse salino, e o segundo capítulo aprofunda a caracterização funcional do gene Os*GPX3* na parte aérea de plantas de arroz, por meio de uma abordagem proteômica, revelando importantes funções desse gene em processos-chave de sinalização e nas vias de resposta do hormônio ácido abscísico.

2. REVISÃO BIBLIOGRÁFICA

2.1 Aplicação de abordagens "ômicas" na genética funcional

Desde o completo sequenciamento dos genomas das duas principais plantas modelo, *Arabidopsis* e arroz, diversas plantas tiveram também seus genomas sequenciados, como Populus, Medicago, tomate, milho, entre outros. O sequenciamento genômico leva a melhores anotações de genes e seus produtos, contribuindo para a compreensão de processos celulares moleculares (BUNNIK; LE ROCH, 2013; MATSUMOTO et al., 2005; RENSINK; BUELL, 2005; STONE-ELANDER et al., 2000; VIJ; TYAGI, 2007)

Para investigar uma função gênica específica na biologia, uma estratégia fundamental é a manipulação da expressão gênica em organismos geneticamente transformadas. Com essas estratégias é possível investigar efeitos da superexpressão, do silenciamento ou do nocaute de determinado gene no desenvolvimento desse organismo e o seu papel em outros processos.

Diferentes abordagens "ômicas" podem ser aplicadas ao estudo em genética funcional, como a genômica, transcriptômica, proteômica, interatoma e metabolômica (MOSA; ISMAIL; HELMY, 2017; ZHUANG et al., 2014). A aplicação dessas abordagens geralmente é feita por meio de duas estratégias. A primeira é quando existe o interesse em acessar diretamente a função de um gene específico. Nesses casos, a aplicação das "ômicas" é feita, por exemplo, em plantas transgênicas para investigar as consequências globais da alteração da expressão de um determinado gene. A segunda estratégia é quando o interesse é saber quais genes-chave estão sendo modulados sob determinada condição, como um estresse, por exemplo. Nesse caso, diferentes ferramentas "ômicas" são aplicadas comparando plantas cultivadas em condição controle ou submetidas a determinado tratamento. Genes candidatos selecionados nessa resposta, podem ser, posteriormente, caracterizados funcionalmente em plantas transgênicas especificamente manipuladas na expressão desses genes.

A genômica consiste na análise da sequência de DNA e é por meio dessas análises que os genomas são sequenciados. Durante muito tempo o sequenciamento do DNA foi dependente do método de Sanger de primeira

geração, baseado na terminação da cadeia e na eletroforese capilar. Desde 2005, o sequenciamento de Sanger foi aos poucos sendo substituído com desenvolvimento de tecnologias de segunda geração (BUNNIK; LE ROCH, 2013). Atualmente, o sequenciamento de segunda geração, baseado no sequenciamento por síntese, tem sido largamente utilizado, sendo capaz de sequenciar genomas completos a partir de pouca quantidade de DNA, identificando mutações, polimorfismos, deleções e eventos de duplicação (HEATHER; CHAIN, 2016). Além disso, essa técnica também tem sido utilizada para o estudo de mecanismos epigenéticos, explorando regiões do DNA diferencialmente metiladas ou modificações em histonas (SOTO et al., 2016).

Além do estudo das sequências genômicas, muitas pesquisas investigam o perfil de expressão gênica comparando tecidos em diferentes condições e como essas sequências são reguladas. A transcriptômica consiste no estudo dos transcritos. Antigamente isso era majoritariamente feito pela técnica de *Northen Blotting* ou pela reação em cadeia da polimerase e transcriptase reversa (RT-PCR), que são restritas a um número limitado de transcritos conhecidos. A técnica do microarranjo também foi e ainda é muito utilizada para avaliar perfis de expressão gênica. O microarranjo consiste em milhares de sondas microscópicas de DNA que estão imobilizadas a uma superfície sólida, e, quando hibridizam com uma amostra de interesse, emitem sinais que são proporcionais ao nível de hibridização entre as amostras e as sondas (BUMGARNER, 2014). Atualmente, a técnica mais utilizada para análise de transcritos é conhecida como RNA-seq, que utilizada as tecnologias de segunda geração para sequenciar transcritos e revelar sua abundância diferencial comparando diferentes condições. Ela pode ser implementada para todos os organismos e além da expressão gênica, a técnica de RNA-seq também pode revelar novos transcritos, novas variações de *splicing* alternativo e regulação de pequenos RNAs não codificantes (KUKURBA; MONTGOMERY, 2015).

Apesar de revelar muitas informações sobre regulação gênica, a transcriptômica nem sempre é a melhor abordagem pois nem sempre há uma correlação direta entra abundância de mRNA e proteína (GREENBAUM et al., 2003; PONNALA et al., 2014). Assim, para mensurar abundância diferencial de proteínas usa-se distintas abordagem proteômica. As proteínas são as unidades

funcionais da célula e são essenciais para entendimento mais amplo dos processos biológicos. Além da quantidade, as proteínas também podem sofrer modificações pós-traducionais, aumentando a complexidade de suas estruturas e funções (DUAN; WALTHER, 2015). Além disso, é possível investigar também interações proteína-proteína por meio do estudo do interactoma, usando técnicas como duplo-híbrido ou complementação bimolecular da fluorescência (BiFC) (KUDLA; BOCK, 2016).

Além de investigar mudanças no acúmulo de genes, transcritos ou proteínas, também é possível analisar o perfil de moléculas como aminoácidos, compostos secundários, açúcares, ácidos graxos, entre outros; com a abordagem da metabolômica. Muitos metabólitos são mais diretamente correlacionados com determinado fenótipo celular do que genes ou proteínas e proporcionam uma leitura funcional mais precisa do estado de uma célula (BUNNIK; LE ROCH, 2013).

2.2 Aplicação da genética funcional na resposta a estresses em plantas

O estudo da resposta de plantas a estresse sempre despertou muito interesse da comunidade científica pois auxilia no desenvolvimento de plantas mais tolerantes a estresses abióticos e resistentes a estresses bióticos. Estresse abióticos como salinidade, seca, alagamentos, altas ou baixas temperaturas, deficiência de minerais ou toxicidade de metais limitam a produtividade agronômica no mundo inteiro (BECHTOLD; FIELD, 2018). Assim, o entendimento dessas respostas é essencial para programas de melhoramento genético de plantas cultiváveis.

Inúmeros trabalhos e revisões têm sido publicados nessa linha, somando esforços pela busca de genes fundamentais nessas respostas (LANGRIDGE, 2006; VIJ; TYAGI, 2007; JAIN, 2015; MOSA; ISMAIL; HELMY, 2017). A **Tabela 1** apresenta alguns exemplos de estudos que exploram a resposta de diferentes plantas aos estresses abióticos em busca de genes candidatos à tolerância.

Tabela 1. Exemplos de estudos que exploraram a resposta de diferentes plantas a estresses abióticos

Espécie	Estresse	Referência
Capsicum annum	Seca	LAKSHMI SAHITYA et al. (2018)
Hordeum vulgare	Salinidade	JAMSHIDI; JAVANMARD (2016)
	Hipoxia	LUAN et al. (2018)
Oryza sativa	Salinidade	PAIVA et al. (2018)
	Seca	SAHEBI et al. (2018)
	Altas temperaturas	CHATURVEDI et al. (2017)
	Excesso de ferro	AUNG et al. (2018)
	Deficiência de nitrogênio	HSIEH et al. (2018)
	Metais pesados	YAMAZAKI et al. (2018)
	Frio	XIAO et al. (2018)
Populus euphratica	Salinidade	CHEN et al. (2017)
Solanum tuberosum	Múltiplos estresses	BAGRI et al. (2018)
Sorghum bicolor	Seca	SPINDEL et al. (2018)
Triticum aestivum	Baixas temperaturas	ZHANG et al. (2018)
	Salinidade	EBEL et al. (2018)

Um dos fatores compartilhados por todos esses estresses listados como exemplo da Tabela 1 é a indução do estresse oxidativo (CHOUDHURY et al., 2017). Dessa forma, considerando sua importância, estudos que busquem um melhor entendimento de como o estresse oxidativo é regulado e quais os principais componentes de suas respostas são de grande relevância.

2.3 Estresse Oxidativo

O estresse oxidativo é um complexo de fenômenos químicos e fisiológicos que resultam na superprodução e acúmulo de espécies reativas de oxigênio (ERO). Esse processo é naturalmente desencadeado nas células vegetais durante as reações aeróbicas, como fotossíntese e respiração, e é agravado quando a planta é submetida tanto a estresses abióticos como bióticos (FALTIN et al., 2010; KIM et al., 2017).

As ERO são definidas como substâncias que contém um ou mais átomos de oxigênio com elétrons desemparelhados, como o radical superóxido ($\cdot O_2$-), radical hidroxila ($\cdot$OH) e o peróxido de hidrogênio (H_2O_2), e são conhecidas mediadoras de cascatas de sinalização intracelular, desempenhando um importante papel na ativação de fatores de transcrição e na modulação da expressão de genes relacionados com a defesa de patógenos e com o desenvolvimento vegetal (MILLER; SHULAEV; MITTLER, 2008; MITTLER et al., 2004).

Apesar de sua importância como molécula sinalizadora, seu acúmulo excessivo pode causar diversos efeitos negativos, modificando biomoléculas como proteínas, carboidratos, lipídeos e ácidos nucleicos, podendo causar a completa perda de função de alguns sistemas fisiológicos e até a morte do organismo (FARMER; MUELLER, 2013; MØLLER; JENSEN; HANSSON, 2007). Uma das principais causas para esse acúmulo excessivo de ERO é a ocorrência de um distúrbio na fisiologia celular normal, devido ao ataque de um patógeno ou submissão a um estresse abiótico, causando um desequilíbrio entre a produção e eliminação desses compostos (DEMIDCHIK, 2015).

As plantas lidam o estresse oxidativo desde que iniciaram a produção de O_2 por meio de CO_2 e H_2O, como os primeiros seres fotossintetizantes há cerca de 3,4 bilhões de anos. O crescente aumento na concentração de O_2 no ambiente acabou direcionando a evolução das espécies e determinando a bioquímica das plantas e animais modernos (DOWLING; SIMMONS, 2009).

Devido a sua característica séssil, as plantas tiveram que desenvolver sofisticados sistemas de defesa e adaptação para sobreviverem a condições adversas. Ao longo da evolução, elas desenvolveram estratégias bioquímicas eficientes que as capacitaram a evitar e amenizar os efeitos negativos do estresse oxidativo.

2.5 Mecanismos vegetais de proteção ao estresse oxidativo e as glutationa peroxidases

As plantas possuem sistemas antioxidantes enzimáticos e não enzimáticos que auxiliam na remoção das ERO (GILL; TUTEJA, 2010). Os sistemas não enzimáticos são inespecíficos para diferentes ERO e são compostos pela glutationa, ácido ascórbico, prolina, betaína, carotenos, flavonoides e entre outros. Os sistemas enzimáticos possuem maior afinidade por essas espécies reativas e são representados pelas enzimas: catalase (CAT), superóxido dismutase (SOD), peroxiredoxina (PRXs), deidroascorbato redutase (DHAR), acorbato peroxidase (APX), glutationa peroxidase (GPX) e entre outras (GILL; TUTEJA, 2010)

As peroxidases estão envolvidas em diversos processos fisiológicos, desempenhando um papéis regulatórios nos processos de transdução de sinais, na parede celular, metabolismo de hormônios, resposta a estresses e crescimento celular (PANDEY et al., 2017). Essas enzimas representam um grande grupo de famílias multigênicas que catalisam a redução do peróxido usando uma variedade de substratos como: lignina, lipídeos de membrana e algumas cadeias laterais de aminoácidos. Devido ao seu papel multifuncional, sua atividade pode ser detectada em todas as etapas da vida de diversas plantas, desde a germinação até a senescência (PASSARDI et al., 2005).

Organelas com uma grande atividade metabólica oxidante ou com grande taxa de fluxo de elétrons, como as mitocôndrias, são importantes fontes na geração de ERO intracelular (RHOADS, 2006). As mitocôndrias, organelas produtoras de energia, são relatadas como um dos principais sítios de produção de ERO (NAVROT et al., 2007; RASMUSSON; SOOLE; ELTHON, 2004), o que ocorre predominantemente durante cadeia transportadora de elétrons pelas NAD(P)H desidrogenases (complexo I) e no complexo citocromo bc1 (complexo III) (MØLLER; JENSEN; HANSSON, 2007), resultando na formação de H_2O_2 principalmente por meio da atividade da enzima superóxido dismutase específica de mitocôndria (RHOADS, 2006). Foi demonstrado recentemente que o complexo II da mitocôndria, a succinato desidrogenase, também é fonte produtora de ERO (JARDIM-MESSEDER et al., 2015). Dentro desse contexto,

se destacam as enzimas antioxidativas presentes nessa organela, como as glutationa peroxidases.

2.6 Função das glutationa peroxidases como atenuadoras de ERO e na transdução de sinais

As glutationa peroxidases (GPXs: EC 1.11.1.9 e EC1.11.1.12) correspondem a um grupo de peroxidases tiol e não heme. Ademais, representam uma família de múltiplas isoenzimas que catalisam a redução de H_2O_2 e outros hidroperóxidos orgânicos em água ou alcoóis correspondentes usando, principalmente, a glutationa reduzida (GSH) como doadora de elétrons em mamíferos e prioritariamente tioredoxina (TRX) em plantas (HERBETTE; ROECKEL-DREVET; DREVET, 2007; TOPPO et al., 2008).

Em plantas, além de estarem envolvidas na homeostase redox, as GPXs também têm papéis em outros processos importantes, como em respostas a estresses bióticos e abióticos e participando de complexas cascatas de sinalização, de forma que a manutenção de sua atividade é essencial para o perfeito funcionamento da célula (GAO et al., 2014; KIM et al., 2014; NAVROT et al., 2006; PAIVA et al., 2018; PASSAIA; MARGIS-PINHEIRO, 2015; RODRIGUEZ MILLA et al., 2003; XU et al., 2012; ZHANG et al., 2012).

Diversos trabalhos já demonstraram que genes GPXs são induzidos em resposta a diferentes estresses abióticos. Em *Capsicum annuum*, a família gênica da GPX foi induzida após estresse com cádmio (LEÓN et al., 2002) e em *Panicum italicum* L. e em *Nicotiana tabacum* após estresse salino (AVSIAN-KRETCHMER et al., 2004; SREENIVASULU et al., 2004) Em *Arabidopsis thaliana* foi demonstrado que essas enzimas têm papel na eliminação de H_2O_2, transdução de sinais, tolerância ao estresse oxidativo e proteção contra danos ao DNA (CHANG et al., 2009; GABER et al., 2012; MIAO et al., 2006).

Existem também relatos que GPXs podem atuar como moduladores redox de outras proteínas, influenciando processos metabólicos críticos (PASSAIA; MARGIS-PINHEIRO, 2015). GPXs e peroxirredoxinas possuem importante papel como sensor redox devido a presença de grupos tióis reativos. A propriedade dessas proteínas de conseguir oxidar proteínas-tiol acaba sendo

muito importante pois permite oxidação de proteínas que não reagem diretamente com peróxido, agindo como intermediários que transferem o sinal redox por diferentes vias (BRIGELIUS-FLOHE; FLOHE, 2011; KLATT; LAMAS, 2000).

Existem relatos de que, em *Oryza sativa,* o gene *PhGPX* aumentou sua expressão após estresse com metais pesados (LI et al., 2000) ao passo que o gene *OsGPX3* após estresse oxidativo (PASSAIA et al., 2013). Além disso, existem evidências de que genes GPX de *Arabidopsis thaliana* estão envolvidos com a percepção de hormônios como ABA e auxina (MIAO et al., 2006) e que vários hormônios como ácido salicílico, ácido abscísico, etileno, auxina e ácido jasmônico afetam a expressão da família de genes At*GPX*, indicando que esses genes estão envolvidos em múltiplas vias de sinalização (GABER, 2014; RODRIGUEZ MILLA et al., 2003).

2.4 *Oryza sativa* e *OsGPXs*

O arroz (*Oryza sativa* L.) é uma angiosperma monocotiledônea pertencente à família Poaceae. Devido seu alto valor nutricional, esta espécie possui grande importância na alimentação humana e na agronomia e, atualmente, representa o alimento com maior potencial para combater a fome no mundo por causa de sua versatilidade e ampla capacidade de adaptação, desempenhando um papel estratégico tanto economicamente como socialmente (Baêta-dos-Santos, 2004).

Durante as últimas quatro décadas, o arroz teve sua produção aumentada em 315% no Brasil, devido ao constante avanço nas tecnologias empregadas em seu cultivo (CONAB, 2017). O Brasil é, atualmente, o nono maior produtor de arroz do mundo (FAOSTAT, 2018), com área plantada de 1,98 milhão de hectares e produção de 12,3 milhões de toneladas do grão na safra 2016/2017 (CONAB, 2017). Sua produtividade média foi de 6224 kg ha^{-1}, a maior desde que a Companhia Nacional de Abastecimento (Conab) passou a monitorar a produção nacional.

Além da sua importância nutritiva e socioeconômica, o arroz é alvo de grandes estudos e é utilizado como planta modelo para estudos fisiológicos,

genéticos e de evolução, principalmente dentro do grupo das monocotiledôneas. Dentre os motivos para ser utilizado como planta modelo, estão o fato de possuir seu genoma completamente sequenciado (MATSUMOTO et al., 2005) ter uma grande similaridade e sintenia com outros importantes cereais como trigo e milho (GALE, 1998; SHIMAMOTO; KYOZUKA, 2002), possuir uma extensa biblioteca de EST disponíveis e por possuir protocolos de transformação por meio da *Agrobacterium tumefaciens* bem estabelecidos (UPADHYAYA *et al*., 2002).

No Brasil, a produtividade do arroz, assim como de outras culturas, depende da capacidade destas de responder a diferentes tipos de adversidades ambientais, os quais geralmente culminam em estresse oxidativo (FALTIN et al., 2010;KIM et al., 2014). Devido a atual conjuntura, é crescente a necessidade de realização de estudos que permitam o entendimento de como as plantas respondem a estresses ambientais. Tendo em vista a grande importância dessa cultura vegetal, a caracterização funcional de genes cujos produtos podem proteger as plantas contra danos oxidativos, como os da glutationa peroxidase, pode, portanto, colaborar com o desenvolvimento de cultivares mais resistentes e adaptadas ao meio ambiente, aumentando sua produtividade e contribuindo na elucidação dos mecanismos envolvidos na interação desses genes com o ambiente. Apesar do estudo funcional dessa classe de enzimas tenha sido iniciado, existem ainda inúmeras lacunas e perguntas biológicas a serem elucidadas.

3. OBJETIVOS

Objetivo geral:

Caracterizar funcionalmente a isoforma mitocondrial do gene de glutationa peroxidase (*OsGPX3*) em arroz.

Objetivos específicos:

- Analisar fenótipo, respostas bioquímicas e fisiológicas de plantas de arroz silenciadas para o gene *OsGPX3* (GPX3s) em resposta ao estresse salino;

- Identificar, na parte aérea e em raízes, proteínas diferencialmente acumuladas quando o gene *OsGPX3* é silenciado e/ou após tratamento com ABA;

-Validar, por RT-qPCR, a expressão diferencial de alguns genes candidatos, tendo como referência os resultados da proteômica;

- Comparar respostas induzidas pelo ABA em plantas NT e GPX3s, como abertura estomática, inibição da germinação, indução de ROS, proteção à seca e indução de senescência;

- Propor mecanismos de atuação do gene *OsGPX3* em arroz.

4.1. CAPÍTULO 1

Para estudar a importância do gene *OsGPX3* nas respostas a estresses abióticos, plantas GPX3s e NT foram submetidas a condições de seca, deficiência de ferro e salinidade. No entanto, apenas na condição de estresse salino que foram observadas diferenças visíveis no fenótipo das plantas. Assim, esse estresse foi escolhido para ser estudado com mais profundidade.

Este capítulo é referente ao artigo "The mitochondrial glutathione peroxidase (*OsGPX3*) has a crucial role in rice protection aginst salt stress", aceito para publicação em outubro de 2018 na revista Enviromental and Experimental Botany. Nesse trabalho descrevemos o gene *OsGPX3* como novo e importante recurso para defesa contra estresse salino em plantas de arroz. Muitos trabalhos já mostraram a importância dessa classe de enzimas na proteção contra o estresse atuando diretamente no metabolismo redox. No

entanto, aqui mostramos que a sensibilidade das plantas GPX3s não ocorre por um acúmulo maior de espécies reativas de oxigênio, revelando novas possibilidades para essas enzimas na proteção contra estresses abióticos.

Capítulo I

A glutationa peroxidase mitocondrial (Os*GPX3*) tem um papel crucial na proteção contra estresse salino em arroz

1 **Mitochondrial glutathione peroxidase (*OsGPX3*) has a crucial role in**
2 **rice protection against salt stress**

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13 **ABSTRACT**

14 Rice is one of the world's most important crops and an excellent model system
15 for understanding the interaction between genes and environmental changes.
16 However, its productivity is often challenged by abiotic stresses, which results in
17 the accumulation of reactive oxygen species. Glutathione peroxidases are part of
18 the mechanism by which plants face oxidative stress. These enzymes can control
19 redox homeostasis and also play a role in redox signaling. Here, we investigate
20 the role of rice GPX3 in plant responses to salt stress using *OsGPX3*-RNAi
21 silenced rice plants (GPX3s). Our results indicate that GPX3s plants are more
22 sensitive to salinity showing decreased biomass, CO_2 assimilation rate, stomatal
23 conductance, and intercellular CO_2 partial pressure. Moreover, these plants
24 present significant damage to photosystem II activity and decline in chlorophyll
25 content. Salt stress induced ROS accumulation in both non-transformed (NT) and
26 GPX3s plants, indicating that GPX3s sensibility to salt stress was not due to the

significant impairment in redox equilibrium. Together, these results show GPX3 importance in rice to achieve salt stress tolerance via an independent ROS-scavenger mechanism. Moreover, it also provides new light into the cross-talk between chloroplasts and mitochondria, suggesting a novel role to this enzyme beyond its role as ROS-scavenger.

Keywords: *Oryza sativa*; Mitochondria; Oxidative stress; Salinity

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops and feeds more than half of the world's population. It is also an excellent model to understand the impact of environmental stresses on crop yield, particularly in the monocotyledon group. Rice productivity in many areas can be affected by different adverse conditions. In this context, the characterization of genes involved in the stress response mechanisms is essential for the development of strategies to promote the improvement of this crop (Shankar et al., 2016; Almeida et al., 2016).

Oxidative stress is a complex of chemical and physiological phenomena that results in reactive oxygen species (ROS) accumulation, leading to macromolecule oxidation and producing adverse effects on cellular metabolism (Foyer, 2018). Plant cells contain a vast array of antioxidant enzymes to attenuate this stress. Glutathione peroxidases control the metabolism of oxidants such as ROS and also play a role in redox signaling (Foyer and Noctor, 2009, 2011).

Rice glutathione peroxidase (*OsGPX*) gene family comprises five members spread throughout a range of subcellular compartments: GPX1 and GPX3 (mitochondria), GPX2 and GPX5 (cytosol and chloroplast) and GPX4

(chloroplast) (Margis et al., 2008; Passaia et al., 2013). This gene family is important to prevent or restrict H_2O_2 accumulation, protect membranes against ROS-induced damage and act in cellular signaling (Foyer and Noctor, 2011).

Different studies have explored the role of GPX isoforms in plant development, such as their importance under abiotic and biotic stresses as well as signal transducer and as redox sensor proteins (Passaia and Margis-Pinheiro, 2015). It has been demonstrated that organelles with high metabolic oxidant activity or high electron flow rate, such as mitochondria, are critical intracellular source of ROS (Turrens, 2003; Stowe and Camara, 2009; Foyer and Noctor, 2011; Jardim-Messeder et al., 2015). In spite of that, the physiological role of plant mitochondrial GPX is still poorly understood.

Previous functional studies of rice mitochondrial isoforms showed that *OsGPX1*-silenced plants have reduced shoot length and seed number compared to non-transformed rice plants (Passaia et al., 2014). It has also been demonstrated that these plants have photosynthesis impairment under normal and salt stress conditions (Lima-Melo et al., 2016). The silencing of the *OsGPX3* gene impairs normal plant development and leads to a stress-induced morphogenic response via H_2O_2 accumulation in young plants (Passaia et al., 2013). However, the importance of this specific gene in plant response to harmful environmental conditions is entirely unknown.

The functional characterization of genes that can protect plants against the harmful effects induced by oxidative stress is crucial for classical breeding and biotechnology programs with the aim to improve stress tolerance. Therefore, salt stress was chosen to evaluate the role of the mitochondrial GPX3 gene in rice

76 plants. The present work characterizes growth, biochemical and physiological
77 traits of non-transformed and GPX3-silenced plants under salt stress.

78

79 **2. MATERIALS AND METHODS**

80 **2.1. Plant growth and treatments**

81 To investigate the role of GPX3 in plant growth and development in control
82 and abiotic stress conditions, non-transformed (NT) (*Oryza sativa* L. ssp.
83 *Japonica* cv. Nipponbare) and GPX3-silenced rice plants were used in this study.
84 Rice *OsGPX3*-silenced plants (GPX3s) were previously generated according to
85 Passaia et al. (2013). Seeds from both genotypes (NT and GPX3s) were
86 germinated in filter paper wet with H_2O at 28 °C in the light, under a 12-h
87 photoperiod. Ten-day-old seedlings were transferred to 2.5 L plastic pots
88 containing Hoagland's solution (Hoagland and Arnon, 1950) and cultivated in a
89 greenhouse with the following environmental conditions: photosynthetic photon
90 flux density (PPFD) varying from 300-650 µmol m^{-2} s^{-1} (190SA quantum sensor,
91 LI-COR, Nebraska USA), photoperiod of about 12 h, temperatures of 27 ± 0.8 °C
92 (night) and 31 ± 3.0 °C (day), and 79.8 ± 10.9% relative humidity. A set of
93 homogenous seeds or plants (four-week-old) were distributed randomly and
94 divided into two groups (control and salt stress).

95 To study the importance of GPX3 during the germination process seeds of
96 NT and GPX3s plants were sown in a filter paper wet with H_2O (control) or 100
97 mM NaCl for seven days. Further, to analyze the GPX3 role in rice growth and
98 development, four-week-old NT and GPX3s plants were exposed to moderate
99 and severe salt stress (100 and 200 mM NaCl, respectively) until eight
100 consecutive days under greenhouse conditions (as described above). The salt-

101 stressed plants were submitted to 100 mM or 200 mM NaCl, added gradually (50
102 mmol NaCl L^{-1} per day) into the nutrient solution to avoid osmotic shock.
103 Photosynthetic parameters including gas exchange and chlorophyll *a*
104 fluorescence were measured at 1 and 3 days after treatment (DAT) in control and
105 100 mM NaCl-treated plants. To quantify the biomass production, photosynthetic
106 pigments and oxidative stress indicators under severe salt stress, plants were
107 exposed to 200 mM NaCl during four days in greenhouse conditions.

108 **2.2. Gas exchange and photochemical parameters**

109 Leaf gas exchange and chlorophyll *a* fluorescence measurements were
110 taken simultaneously using a portable Infra-red Gas Analyzer coupled with a leaf
111 chamber fluorometer (Li-6400-XT, LI-COR, Lincoln, NE, USA) in plants
112 previously acclimated to growth light conditions (3 h). During the measurements
113 the environmental conditions inside the IRGA's chamber were: leaf temperature
114 of 28 ºC, PPFD of 1,000 μmol m^{-2} s^{-1}, 1.8 ± 0.2 kPa vapor pressure deficit (VPD)
115 and CO_2 partial pressure of 38 Pa. The amount of blue light was set to be 10% of
116 the PPFD to maximize stomatal aperture (Flexas et al. 2004). The gas exchange
117 parameters measured were: net photosynthesis (P_N), stomatal conductance (g_s),
118 transpiration rate (E) and intercellular CO_2 partial pressure (Ci).

119 The fluorescence parameters were measured using the saturation pulse
120 method in light leaves (Schreiber et al., 1995). The intensity and duration of the
121 light saturation pulse were 8,000 μmol m^{-2} s^{-1} and 0.7 s, respectively. The
122 photochemical parameters of PSII assessed were: the effective quantum
123 efficiency of PSII [ΦPSII = (Fm´−Fs)/Fm´] and the apparent electron transport
124 rate of PSII (ETR = (ΦPSII×PPFD×0.5×0.84), where 0.5 is the presumed fraction
125 of the excitation energy distributed to PSII and 0.84 is the assumed fraction of

126 light absorbed by the leaf (Schreiber et al., 1998). The Fm´ and Fs are the
127 maximum and steady-state fluorescence in the light adapted leaves, respectively
128 (Schreiber et al., 1995).

129 The instantaneous carboxylation efficiency (P_N/Ci) was estimated
130 according to Zhang et al. (2001). The ETR/P_N ratio was also calculated to
131 estimate the use of electrons in other processes not related to the photosynthetic
132 CO_2 assimilation (Ribeiro et al., 2009).

133

134 **2.3. Biomass and photosynthetic pigment determination**

135 The whole plant biomass (shoots and roots fresh weight) was analyzed
136 using five randomly selected plants of each genotype. The plants were weighed
137 four days after 200 mM NaCl treatment. Total chlorophyll ("a" and "b") and
138 carotenoid contents were determined after extraction in ethanol and measured
139 spectrophotometrically at 665 and 649 nm and 475, respectively. The amount of
140 these pigments was calculated using the equations proposed by Lichtenthaler
141 and Wellburn (1983).

142

143 **2.4. Lipid peroxidation determination**

144 The level of lipid peroxidation was estimated by the method of Heath and
145 Packer (1968) in term of MDA content determined by thiobarbituric acid (TBA)
146 reaction. 200 mg of fresh tissue was homogenized with 5 ml 0.25% TBA
147 containing 10% TCA. The homogenate was boiled for 30 min at 95°C and
148 centrifuged at 10,000g for 10 min. The absorbance of the supernatant was
149 recorded at 532 nm and corrected by subtracting absorbance at 600 nm. The
150 amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

151 **2.5. ROS measurement**

152 To measure H_2O_2 shoot and roots from control and salt-treated material (1
153 g) were immersed in liquid nitrogen and finely grounded to a powder with a mortar
154 and pestle and after homogenized with 2 mL of 100 mM K-phosphate buffer (pH
155 6.8, containing 0.1 mM EDTA). After centrifugation at 12,000 × g, 15 min, 4 °C,
156 the supernatant (crude extract) was kept and total soluble proteins were
157 quantified using the Bradford (1976) method, and subsequently H_2O_2 content was
158 quantified using the Ampliflu Red (Sigma-Aldrich) oxidation method (Smith et al.,
159 2004). The crude extract was incubated with 10 mM Ampliflu Red and five units
160 ml^{-1} horseradish peroxidase and the fluorescence monitored using a fluorimeter
161 at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5
162 nm), respectively. For histochemical detection of ROS and superoxide anion (O_2
163 $^-$) H_2DCFDA (2′,7′-Dichlorodihydrofluorescein diacetate) and NBT (nitro blue
164 tetrazolium) staining were respectively used. The leaves segments were
165 incubated in 10 μM H2DCFDA and were vacuum infiltrated for 5 min. The leaves
166 were washed with double distilled water and observed under confocal microscope
167 using laser beam excitation at 488 nm (Kristiansen et al., 2009). For NBT assay,
168 the leaf segments were immersed and vacuum infiltrated with 0.1% (w/v) nitro-
169 blue tetrazolium (NBT) staining solution in 10 mM potassium phosphate buffer
170 (pH 7.8) staining solution containing 10 mM NaN_3 for 30 min at room temperature.
171 Stained leaves were bleached in 0.15% (w/v) trichloroacetic acid in
172 ethanol:chloroform (4:1 v/v) at 100°C for 30 min, and stored in glycerol:ethanol
173 (1:4 v/v) solution until photographed (adapted from Rao et al., 1999).

174

175

176 **2.6. Quantitative real-time PCR (RT-qPCR)**

177 Real-time PCR experiments were carried out using cDNA from leaves
178 synthesized from total RNA purified with TRIzol (Invitrogen®). Synthesis of the
179 first-strand cDNA was performed by incubating 1 μg of total RNA with the M-MLV
180 Reverse Transcriptase (Promega) and a 24-polyTV primer (Invitrogen®). After
181 synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR
182 reactions. Four biological replicates of each genotype under both conditions,
183 control and four-days after 200 mM NaCl treatment, and three technical replicates
184 were performed for each reaction. All data analyses were performed after
185 comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method
186 (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). RT-qPCR reactions
187 were performed in an Applied Biosystems StepOne plus Real Time PCR system
188 (Applied Biosystems ®) using SYBR. The primers used as internal controls to
189 normalize the amount of mRNA present in each sample were *OsFdh3* and
190 *OsEif1a* and the targets were *OsGPX* genes. All primers' sequences are listed in
191 Passaia et al. (2013).

192

193 **2.6. Statistical Analysis**

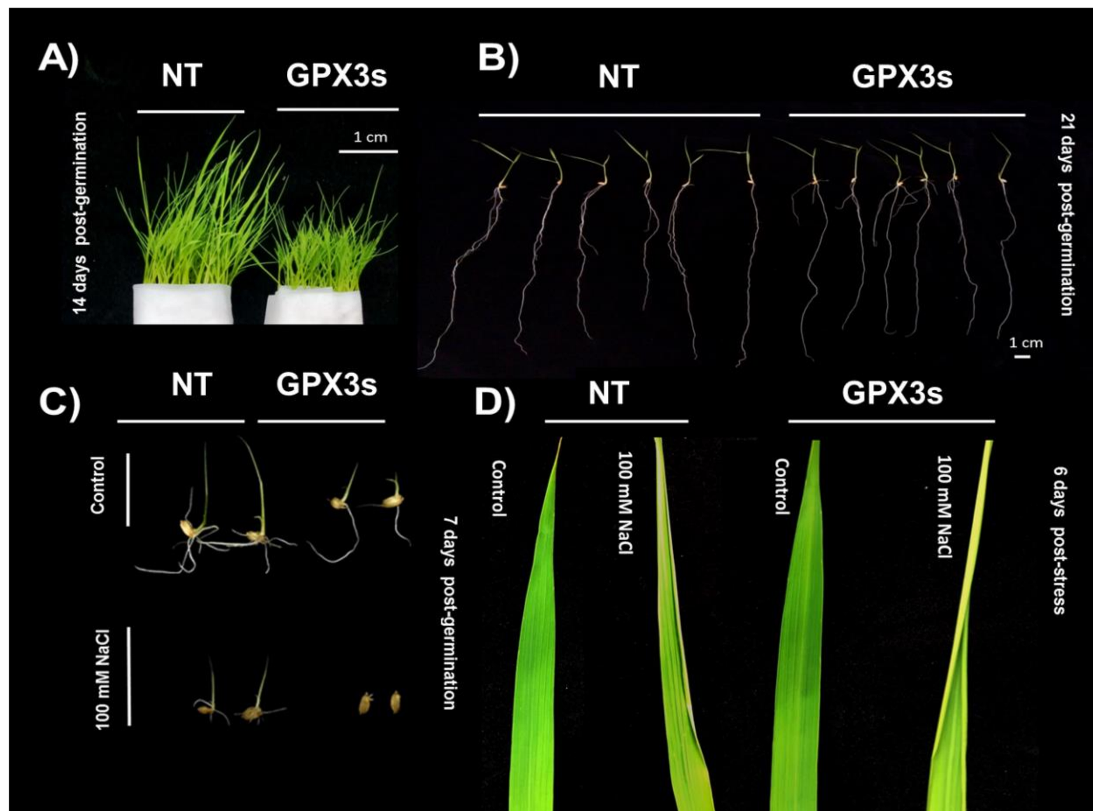
194 The experiments were arranged in a completely randomized design in a
195 2x2 factorial: two genotypes (NT and GPX3s) and two treatments (control and
196 salinity). Four biological replicates were used to gas exchange and
197 photochemical parameters analyzes, using individual pot containing 2 plants
198 represented each replicate, while five biological replicates were used to pigment
199 content, biomass, lipid peroxidation and ROS measurements. Data were plotted
200 with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and

201 analyzed using Two-way ANOVA followed by Tukey’s multiple comparisons test.
202 Data were considered statistically significant at $p \leq 0.05$. Uppercase letters are
203 used just in graphics with more than one time-point and represent statistically
204 significant differences ($p \leq 0.05$) comparing plants at 1 DAT and 3 DAT.
205 Lowercase letters denote statistically significant differences among NT and
206 GPX3s plants, in the same time of exposure, under control or salt conditions.
207
208 **3. RESULTS**

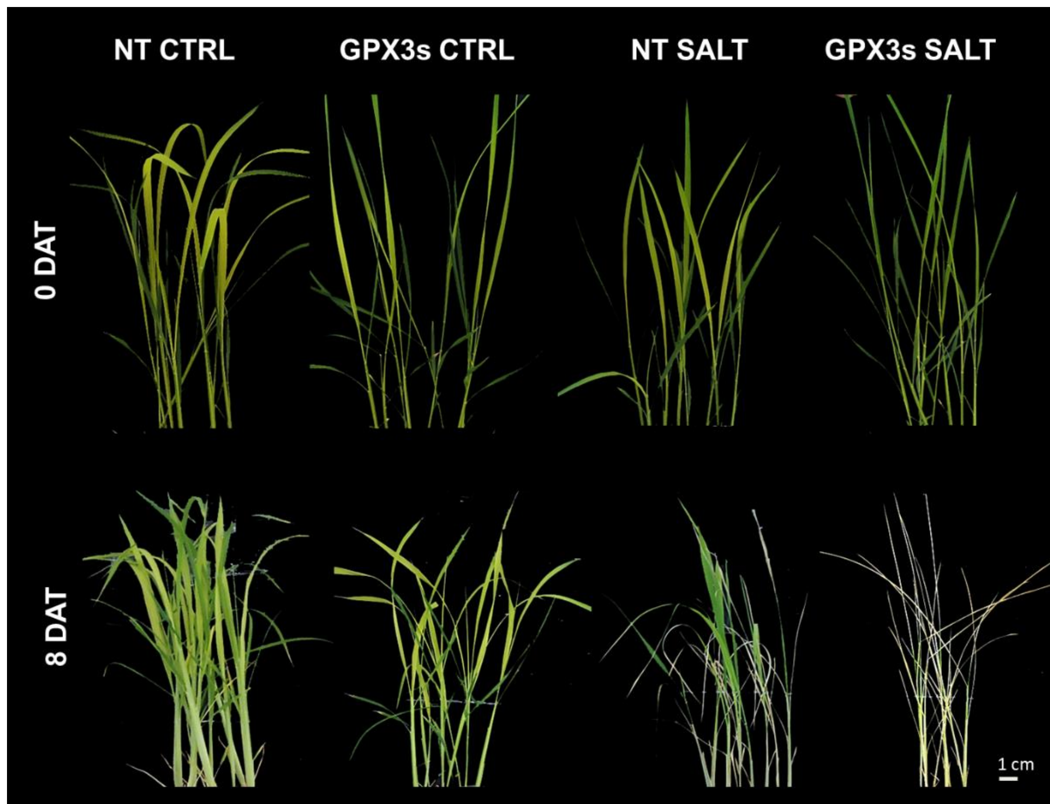
209 **3.1 Phenotypic characterization of Os*GPX3*-silenced plants under salt**
210 **stress**

211 To better understand the GPX3 function in rice seed germination and
212 growth under abiotic stress, seeds of non-transformed and GPX3s plants were
213 cultivated under control or salt stress conditions. GPX3-silenced plants (GPX3s)
214 displayed a germination delay and slower growth compared to NT plants up to 14
215 days post-germination (**Fig. 1A**). However, this difference disappeared in later
216 developmental stages (21 days post-germination) (**Fig. 1B**). GPX3s seeds also
217 presented a severe germination delay under salt stress conditions (**Fig. 1C**).

218 Our results also show that salt stress induces damage in NT plants and
219 this negative impact was visually more pronounced in transgenic plants. GPX3s
220 plants exhibited more stress symptoms in leaves (dehydrated and chlorotic) than
221 NT plants, after 6 days of 100 mM NaCl exposure, suggesting that GPX3s are
222 more sensitive to salt stress than NT plants (**Fig. 1D**). This result was reinforced
223 when 4-week-old plants under 200 mM NaCl also revealed a strong sensibility to
224 high salinity conditions (leaves completely chlorotic and dehydrated) (**Fig. 2**).

225 

226 **Figure 1**. Phenotypic characterization of representative non-transformed (NT)
227 and GPX3s rice plants under control or salt stress (100 mM NaCl) conditions. (A)
228 Seedling leaves at 14 days post-germination and (B) at 21 days post-germination
229 under control conditions (H_2O); (C) Seed germination six days after sown under
230 control (H_2O) or 100 mM NaCl conditions; (D) Leaves of 4-week-old plants after
231 six days under control or 100 mM NaCl conditions in greenhouse.



239

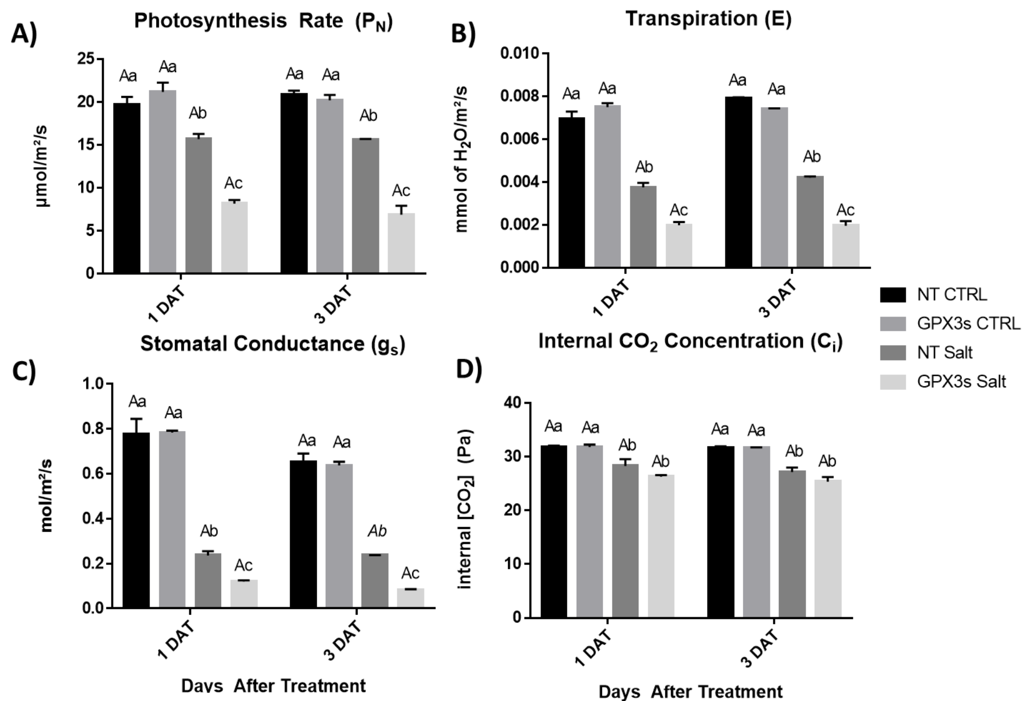
240 **Figure 2**. Phenotypic effect on shoots of 4-week-old non-transformed (NT) and
241 GPX3s plants under control or salt stress (200 mM NaCl) conditions. Pictures
242 were taken before salt stress (0 days after treatment) and 8 days after treatment.

243

244 **3.2 Photosynthetic parameters, pigments content and biomass**

245 To further analyze the role of GPX3 in rice plant growth, photosynthetic
246 parameters, pigment content and biomass production were measured under
247 moderate (100 mM NaCl) and severe (200 mM NaCl) salt stress conditions. Leaf
248 gas exchange in NT and GPX3s plants was measured at one and three days after
249 treatment (DAT), starting when final NaCl concentration was achieved (**Fig. 3**).
250 Under control conditions, gas exchange parameters (net photosynthesis (P_N),
251 transpiration rate (E), stomatal conductance (g_s) and internal CO_2 partial pressure
252 (C_i)) were similar in both genotypes (1 and 3 DAT). However, when plants were

253 treated with 100 mM NaCl, P_N, E and g_s decreased in both plants, but it was
254 more effective on GPX3s plants in both 1 and 3 DAT (**Fig. 3A, B and C**).
255 Differently, C_i reduced in the same proportion in both genotypes and days (1 and
256 3 DAT) under salt stress regarding to their respective controls (**Fig. 3D**).

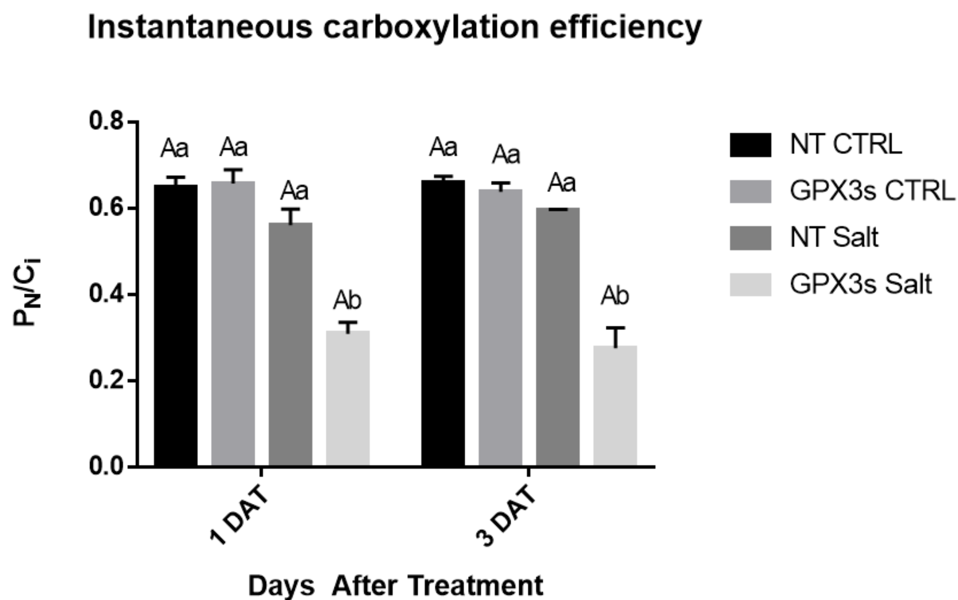


257

258 **Figure 3.** Leaf gas exchange parameters in non-transformed (NT) and GPX3s
259 rice plants under control or salt stress (100 mM NaCl) conditions. Measurements
260 were performed at 1 and 3 days after treatment. (A) Net photosynthesis, (B)
261 transpiration rate, (C) stomatal conductance and (D) internal CO_2 partial
262 pressure. Each bar represents the average of four replicates ± SEM. Data were
263 analyzed using Two-way ANOVA followed by Tukey's multiple comparisons test.
264 Different uppercase letters represent statistically significant differences ($p \leq 0.05$)
265 comparing genotypes at 1 DAT and 3 DAT, while different lowercase letters
266 represent statistically significant differences among NT and GPX3s plants, in the
267 same time of exposure, under control or salt conditions.

268 Measurements of the relationship between photosynthesis (P_N) and
269 internal CO_2 partial pressure (C_i) represent the instantaneous carboxylation

270 efficiency, which can be linked with Rubisco carboxylation *in vivo* activity. As
271 observed before, P_N/C_i did not change between the genotypes under control
272 conditions, but it was severely decreased in GPX3-silenced plants under salt
273 stress similarly at 1 DAT and 3 DAT (**Fig. 4**).



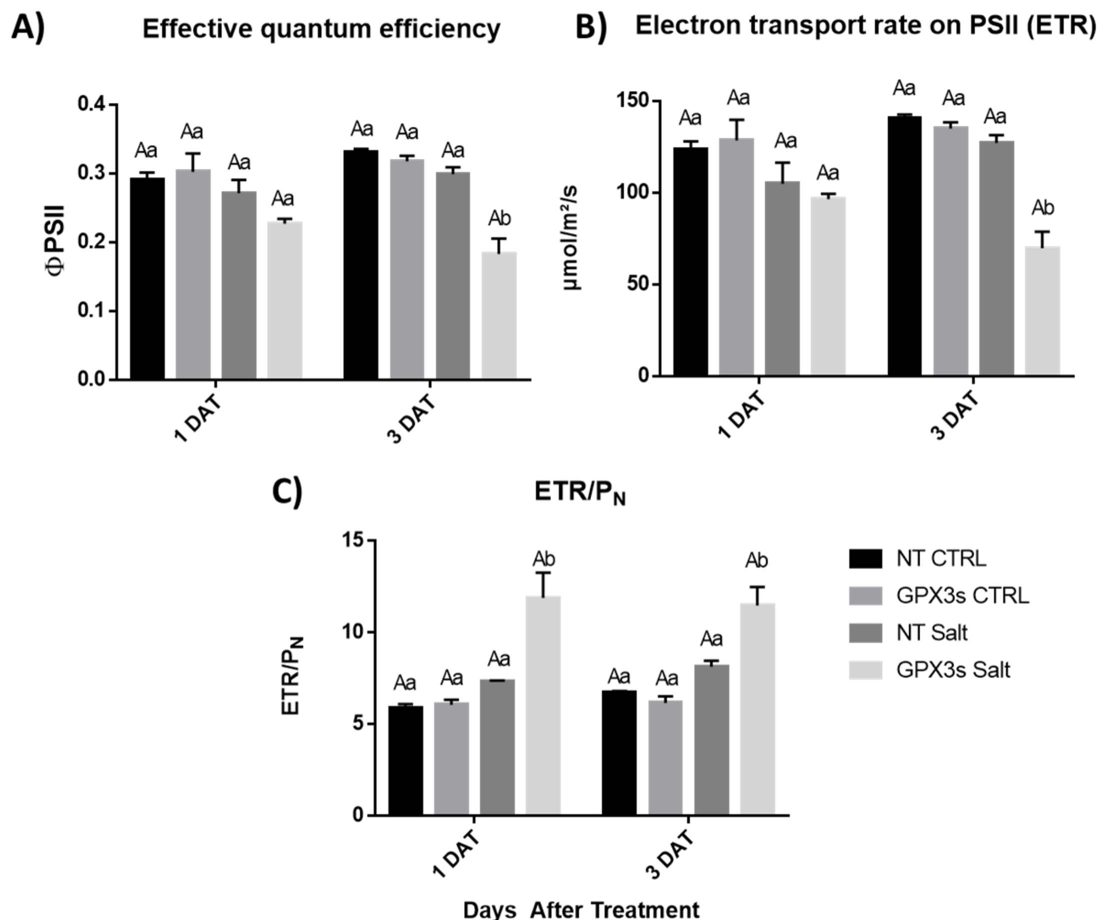
274

275 **Figure 4**. Instantaneous carboxylation efficiency (P_N/C_i) in non-transformed (NT)
276 and GPX3s rice plants under control or salt stress (100 mM NaCl) conditions.
277 Measurements were performed at 1 and 3 days after treatment. Each bar
278 represents the average of four replicates ± SEM. Data were analyzed using Two-
279 way ANOVA followed by Tukey's multiple comparisons test. Different uppercase
280 letters represent statistically significant differences ($p \leq 0.05$) comparing
281 genotypes at 1 and 3 days after treatment, while different lowercase letters
282 represent statistically significant differences among NT and GPX3s plants, in the
283 same time of exposure, under control or salt conditions.

284

285 Chlorophyll *a* fluorescence parameters were also assessed to analyze the
286 effects of salt stress in the electron transport chain in the thylakoid membrane.

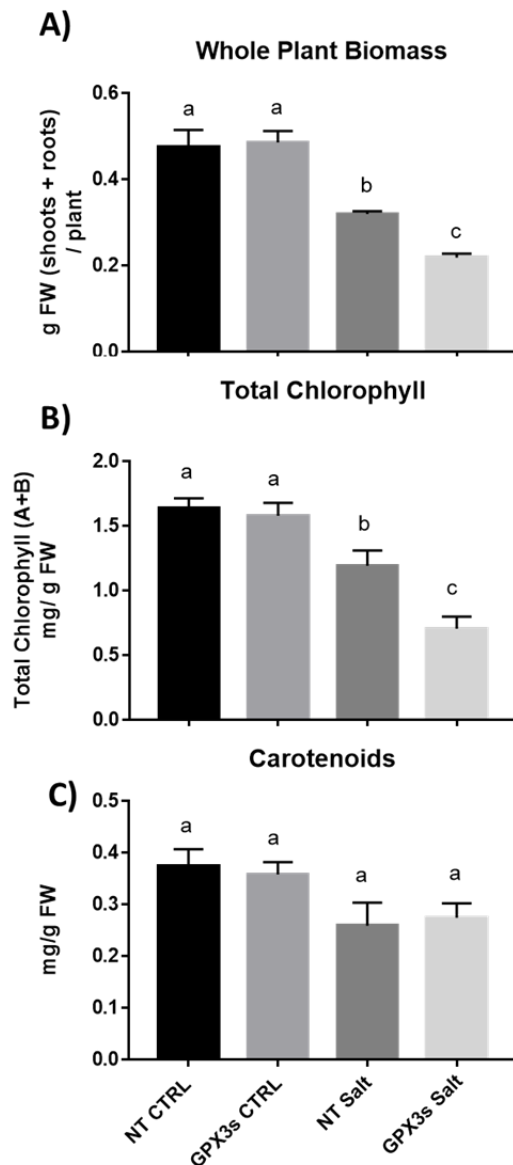
287 There were no statistical differences between genotypes under control conditions
288 for the photochemical parameters. However, under salinity, the effective quantum
289 efficiency and electron transport rate of PSII (ΦPSII and ETR, respectively)
290 decreased in GPX3s at 3 DAT and did not change in NT plants when compared
291 with their respective controls (**Fig. 5A and B**). Contrarily, the ETR/P_N ratio was
292 highly increased in GPX3-silenced plants after 1 DAT and 3 DAT and did not
293 change in NT-stressed plants (**Fig. 5C**).



294 **Figure 5**. Salt treatment effect on chlorophyll *a* fluorescence parameters. (A)
295 effective quantum efficiency of PSII, (B) electron transport rate of PSII and (C)
296 ETR/P_N ratio in non-transformed (NT) and GPX3s rice plants under control or salt
297 stress (100 mM NaCl) conditions. Measurements were performed at 1 and 3 days
298 after treatment. Each bar represents the average of four replicates ± SEM. Data
299 were analyzed using Two-way ANOVA followed by Tukey's multiple comparisons

300 test. Different uppercase letters represent statistically significant differences ($p \leq$
301 0.05) comparing genotypes at 1 and 3 days after treatment, while different
302 lowercase letters represent statistically significant differences among NT and
303 GPX3s plants, in the same time of exposure, under control or salt conditions.

304 The biomass production and photosynthetic pigment contents were
305 assessed in four-day 200 mM NaCl-treated and control NT and GPX3s plants.
306 Biomass fresh weight reduced by 33% in NT and 53% in GPX3s when compared
307 with control plants **(Fig. 6A)**. This response was corroborated with reductions in
308 total chlorophyll (a+b) content in both NT- and GPX3s-salt-treated plants, but this
309 parameter was much lower (50% less) in the transformed plants after salt
310 exposure **(Fig. 6B)**. In contrast, the concentration of carotenoid did not change in
311 any of genotypes and treatments **(Fig. 6C)**.



312

313 **Figure 6** Effect of salt treatment on 4-week-old non-transformed (NT) and GPX3s
314 plants exposed to 200 mM NaCl for four days. (A) whole plant biomass; (B) total
315 chlorophyll; (C) carotenoid content. Each bar represents the average of five
316 replicates ± SEM. Data were analyzed using Two-way ANOVA followed by
317 Tukey's multiple comparisons test. Different letters represent data considered
318 statistically significant at $p \leq 0.05$.

319 **3.3 Oxidative stress parameters**

320 To investigate if GPX3s susceptibility to salt stress is directly related to
321 oxidative stress, we characterized quantitatively and qualitatively ROS

322 accumulation and lipid peroxidation (TBARS) in both NT and GPX3s plants under
323 control and salt stress (200 mM NaCl) conditions. The lipid peroxidation
324 increased in GPX3s plants under control conditions, as shown by higher
325 malondialdehyde (MDA) contents, when compared to NT plants **(Fig. 7A)**. This
326 oxidative stress indicator also increased after four days of salt supply similarly in
327 both NT and GPX3s plants **(Fig. 7A)**. Our results showed that *OsGPX3* silencing
328 and salt treatment do not change H_2O_2 accumulation significantly in shoots (**Fig.
329 7B**). However, a lower H_2O_2 content under control conditions was verified in
330 GPX3s roots regarding NT plants (**Fig. 7C**). On the other hand, a H_2O_2 content
331 increase of 71.8% and 98.1% was observed for NT and GPX3s roots under salt
332 stress, respectively, when compared to their respective controls (**Fig. 7C**).
333 Significant differences were not identified between genotypes under salt stress
334 (**Fig. 7C**).

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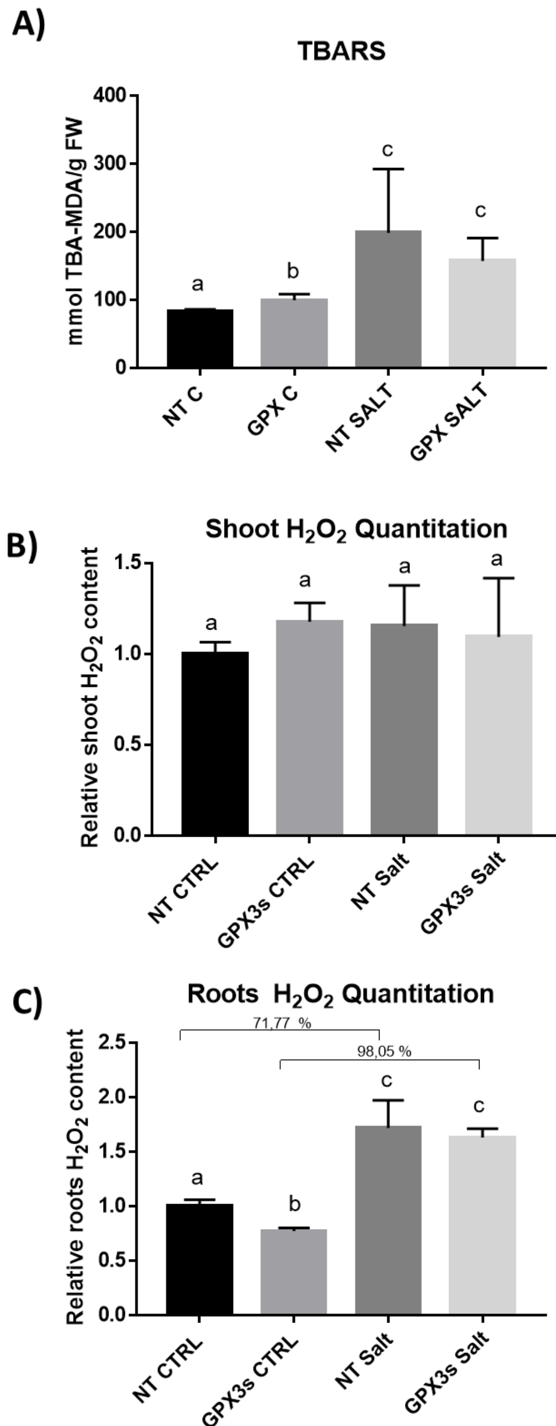
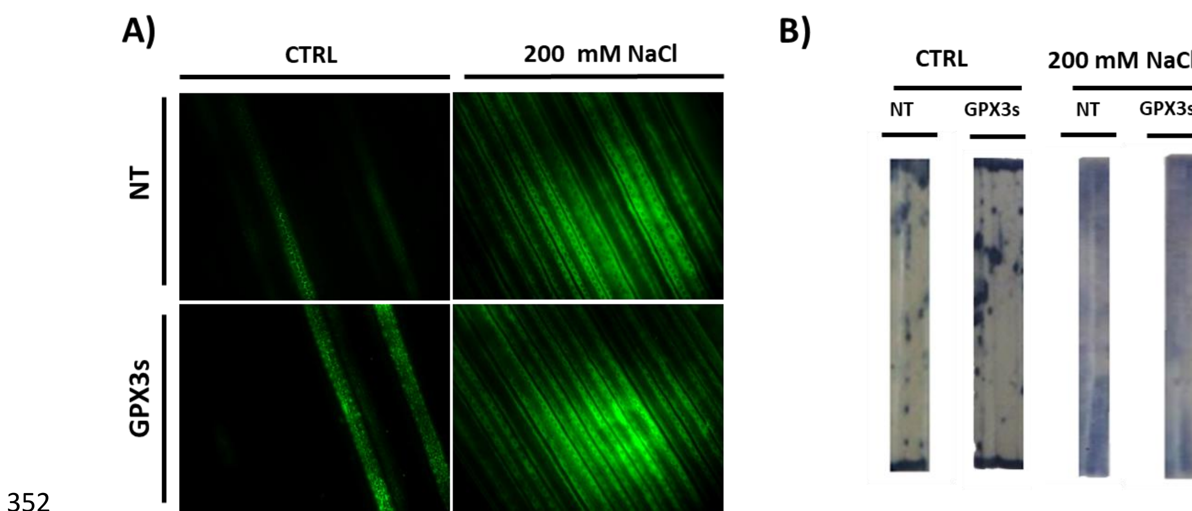


340

341 **Figure 7**. Quantitative oxidative stress indicators in non-transformed and GPX3s
342 rice plants cultivated in control or salt stress. Measurements were performed after
343 four days of salt supply (200 mM NaCl). (A) Thiobarbituric acid reactive
344 substances (TBARS) content in leaves and H_2O_2 content in (B) shoots and (C)
345 roots. Each bar represents the average of five replicates ± SD. Data were

346 analyzed using Two-way ANOVA followed by Tukey's multiple comparisons test.
347 Different letters represent data considered statistically significant at $p \leq 0.05$.

348 In leaves, a similar result was found with H_2DCFDA and NBT superoxide
349 anion-staining **(Fig. 8)**. These results show that salt induces ROS accumulation
350 equally in NT and GPX3s plants. Therefore, it does not explain GPX3s sensibility
351 to salt stress.

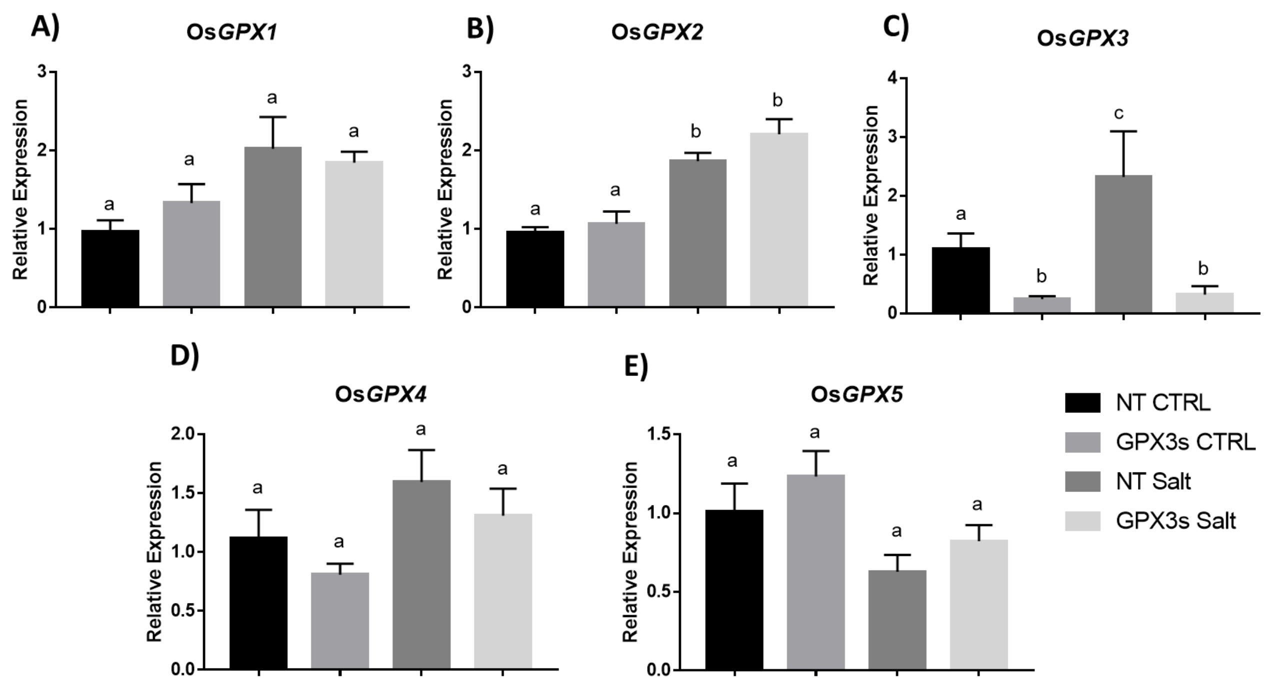
352 

353 **Figure 8**. Leaf qualitative oxidative stress indicators in non-transformed and
354 GPX3s rice plants cultivated in control or salt stress. Measurements were
355 performed after four days of salt supply (200 mM NaCl). (A) Reactive oxygen
356 species content using 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA)
357 staining and (B) superoxide anion content using nitro blue tetrazolium (NBT)
358 staining.

359 **3.4. GPX gene expression**

360 To compare the rice GPXs gene expression in NT and GPX3s plants and
361 to investigate how salt stress modulates these genes, we quantified mRNA of all
362 rice GPX members under control conditions and four-days after NaCl treatment
363 using a RT-qPCR.

364 The results showed that *OsGPX1, OsGPX4* and *OsGPX5* gene expression
365 did not change after GPX3 silencing or after salt stress (**Fig. 9A, D** and **E**).
366 *OsGPX2* was induced after salt stress similarly in NT and GPX3s plants (**Fig.**
367 **9B**). *OsGPX3* mRNA level in GPX3s was reduced to 25% of the control NT plants
368 (**Fig. 9C**), confirming the silencing of this gene in these transgenic plants. On the
369 other hand, *OsGPX3* gene was induced in NT plants after salt stress, indicating
370 an important role of this gene during salt response (**Fig. 9C**).
371



372

373 **Figure 9**. Transcript level of all *OsGPX* genes in non-transformed (NT) and
374 GPX3s plants cultivated in control or salt stress. (A) *OsGPX1*; (B) *OsGPX2*; (C)
375 *OsGPX3*; (D) *OsGPX4* and (E) *OsGPX5*. Measurements were performed after
376 four days of salt supply (200 mM NaCl). The transcript level of *OsGPXs* gene in
377 NT plants under control conditions was used to normalize transcript accumulation
378 in GPX3s and NT plants. The values represent the means ± SEM (n = 4). Each
379 bar represents the average of five replicates ± SEM. Data were analyzed using
380 Two-way ANOVA followed by Tukey's multiple comparisons test. Different letters
381 represent data considered statistically significant at $p \leq 0.05$.

4. DISCUSSION

Our results suggest that *OsGPX3* has an essential role during seed germination, and also for plant growth and development under normal and stressful conditions. Although Passaia et al. (2013) have demonstrated that *OsGPX3* gene silencing generated shorter roots and shoots in an initial-stage rice compared to NT plants, our results indicate that these differences did not persist in later developmental stages. Beside the role of *OsGPX3* in the initial stages of rice plant development, the importance of this antioxidant enzyme in response to stress has not been evaluated until now. Our data show for the first time that several physiological effects occur in GPX3s in response to salt stress, such as lower photosynthesis and biomass.

These metabolic disorders clearly suggest that GPX3s plants are more sensitive to salt stress than NT plants and that this mitochondrial isoform is essential to plant normal growth and development. Rice plants have an efficient antioxidant system composed by many peroxidases isoforms present in all cell compartments (Margis et al., 2008; Lazzarotto et al., 2015). Some of these antioxidant proteins have been deeply studied in plants exposed to abiotic stresses, such as ascorbate peroxidases isoforms and catalase (Castro et al., 2018; Cunha et al., 2018; Jardim-Messeder et al., 2017; Ribeiro et al., 2017; Bonifácio et al., 2016; Sousa et al., 2015). These authors have demonstrated that knockdown expression of APX isoforms (APX1/2, APX4, and APX7/8) in rice did not change plant phenotype, probably because the activity reduction of these isoforms is compensated by alternative routes. Nevertheless, the specific function of GPX3 isoform in plant redox metabolism and retrograde signaling is poorly understood so far.

407 In our study, 4-week-old GPX3-silenced plants exposed to moderate and
408 severe salt stress exhibited strong inhibition of photosynthetic capacity with
409 restrictions on PSII photochemical activity and CO_2 assimilation rate. This later
410 decreased mainly by metabolic limitations, as stomatal conductance decreased
411 and the P_N/C_i ratio increased (Flexas et al., 2004). Meloni et al. (2003) observed
412 failure in the photosynthetic efficiency of cotton plants exposed to salt due to
413 stomatal limitation. Shimazaki et al. (2007) reported that CO_2 assimilation from
414 external medium induces water loss and the decrease in this loss also restricts
415 CO_2 input, leading to a decline in transpiration. The reduction in stomatal
416 conductance reflects less transpiration and consequently less water loss, which
417 increases resistance to abiotic stresses.

418 This study also suggests that the decrease in P_N/C_i ratio is probably related
419 to the drop in ATP and NADPH production, which is needed for CO_2 fixation in
420 the Calvin-Benson cycle (Farquhar and Sharkey, 1982; Shimizu et al., 2015). The
421 decrease of P_N/C_i ratio is possibly connected with Rubisco carboxylase activity
422 reduction and excessive accumulation of salt ions (Na^+ and Cl^-) in shoot tissues.
423 In orange and sorghum, the reduction of photosynthesis was linked with increase
424 of salt ion concentration on the photosynthetic apparatus (López-Climent et al.,
425 2008; Netondo et al., 2004; Silva et al., 2011).

426 Plant survival and performance in response to any stress, including
427 salinity, can be quickly evaluated by chlorophyll fluorescence parameters or
428 pigment contents, given that these conditions induce damage to PSII reaction
429 centers (Woo et al., 2008). Two of the most important chlorophyll *a* fluorescence
430 parameters are the effective quantum efficiency of PSII (ΦPSII) and the electron

431 transport rate of PSII (ETR), that are commonly affected under stress conditions.
432 The increase in ETR/P_N ratio represents an unbalance between electron flow and
433 CO_2 fixation during photosynthesis, and it is frequently associated with increase
434 in electron flow to other physiological processes not linked to Rubisco activity
435 (Ribeiro et al., 2009). During salt stress, there is a decrease in CO_2 fixation,
436 resulting in the inhibition of PSII electron transport, as indicated by the decline in
437 ΦPSII. However, this reduction was not intense as that observed in P_N, indicating
438 other alternative routes, such as photorespiration and water-water cycle acting
439 on excess energy elimination (Tourneux and Peltier, 1995; Huang et al., 2016).

440 The plant photochemistry deficiency due to exposure to salt stress may be
441 linked to possible injuries in primary electron acceptors, which generally occurs
442 under severe oxidative damage (Foyer and Noctor, 2000; Chagas et al., 2008).
443 Total chlorophyll (a+b) content was also markedly reduced by salt treatment in
444 NT and GPX3s plants. These results imply a considerable loss of energy by
445 chlorophyll complex, which can be related to the higher difficulty of plants to
446 absorb water and optimize the process of light energy usage. These findings may
447 be associated with decreased stomatal conductance and CO_2 assimilation rate
448 observed in rice plants at 3 DAT, therefore leading to the sensitivity to salinity.
449 Abiotic stress commonly induces alterations in the thylakoid membrane integrity
450 of chloroplasts with frequent changes in fluorescence signal parameters (Baker
451 and Rosenqvst, 2004). Chlorophyll content reduction could be due to the
452 oxidative stress, Chl photodamage or chlorophyllase activity induced by the salt
453 (Ashraf 2003; Hossain et al. 2011). Higher sensitivity to salinity could be
454 associated with the inability to maintain the higher Chl content and P_N, to better

455 compartmentalize Cl^- and Na^+ in leaves, and to better retain nutrient uptake even
456 in elevated salt concentrations (Kosová et al., 2013; Rodziewicz et al., 2014).

457 The redox homeostasis equilibrium is also essential to maintain membrane
458 integrity and to reduce toxic ion accumulation, which, in turn, decreases
459 photosystem damage. However, some examples showed that H_2O_2 does not
460 always play a primary role in salt stress (Lima-Melo et al., 2016). Our results also
461 indicate that higher salt sensitivity of GPX3s is not directly related with an
462 imbalance in redox metabolism, given that both NT and GPX3s did not have a
463 significant difference in ROS accumulation after salt stress. Nevertheless, GPX3s
464 plants suffered more damage and were more susceptible than NT plants. Passaia
465 et al. (2013) showed that the *OsGPX3* gene silencing did not change the
466 expression of other *OsGPX* isoforms, except *OsGPX2*, which was
467 downregulated, confirming that there is no induction of others GPXs as a
468 compensative mechanism. Our present results confirmed that other rice GPX's
469 members are not induced after *OsGPX3* silencing. However, it cannot be ruled
470 out that the silencing of GPX3 induced ROS accumulation in a specific organelle,
471 which could not be enough high to be detected when the entire shoot or root is
472 quantified. The silencing of the *OsGPX1* in rice plants also led to photosynthetic
473 impairment under normal conditions and this damage was aggravated after salt
474 exposure, despite the fact that H_2O_2 did not change (Lima-Melo et al., 2016).
475 Moreover, there are many other molecules that could be involved in redox
476 equilibrium, as the proteinaceous thiol members such as thioredoxin,
477 peroxiredoxin, and glutaredoxin (Kapoor et al., 2015). Most of the plant's GPX
478 use thioredoxin as a reducing agent rather than glutathione (Navrot et al., 2006)
479 and the mitochondrial thioredoxin activity, for example, was reported being

480 important to plant protection under salinity, stimulating the activities of
481 antioxidative enzymes in *Pisum sativum* (Marti et al., 2011). So, it is crucial to
482 consider the complexity of these interactions in redox metabolism.

483 The effect of *OsGPX3* silencing on photosynthesis parameters
484 corroborates previous reports that a crosstalk between organelles occurs.
485 Hydrogen peroxide can act as a signaling molecule modulating photosynthesis
486 (Bonifacio et al. 2011; Carvalho et al. 2014; Sousa et al. 2015). Some authors
487 reported that this crosstalk between organelles could be mediated by H_2O_2,
488 glutathione and probably other signaling molecules (Munné-Bosch et al. 2013).
489 Thus, mitochondrial redox homeostasis perturbation induced by GPX3 deficiency
490 could trigger changes in mitochondrial-chloroplast interactions, influencing
491 photosynthetic parameters. The importance of mitochondria mitigating adverse
492 effects in chloroplasts was proposed. Mitochondria appear to act by receiving and
493 oxidizing the excess of reducing equivalents generated in stroma using
494 respiratory electron transport chains. Thus, this organelle contributes to maintain
495 photosynthesis efficiency by alleviating electron pressure in chloroplasts
496 (Schreibe et al. 2005; Noctor et al. 2007; Araújo et al. 2014; Lima-Melo et al.,
497 2016).

498 Alternatively, GPX3 may play a role in signaling processes, beyond its
499 function as a ROS scavenger; GPX3 could change plant redox status not by ROS
500 accumulation, but by interacting and modifying other proteins or signaling
501 molecules. Our group found evidence of the possible role of *OsGPX3* in
502 epigenetic regulation and DNA methylation (unpublished data). In Arabidopsis,
503 it was shown that APX1 and GPX1 can induce histone modification by interaction

504 with PRMT4b, playing an important role in oxidative stress tolerance (Luo et al.,
505 2016). It was also illustrated in Arabidopsis that GPX3 can interact directly with
506 ABI2, mediating ABA and oxidative signaling (Miao et al., 2006). Bela et al. (2015)
507 also discussed the GPX role in cellular redox homeostasis maintenance by
508 regulation of thiol/disulfide or NADPH/$NADP^+$ ratio, beyond H_2O_2 detoxification.

509 Further, by controlling redox metabolism, it is also possible that GPX3
510 could act as a redox modulator of other proteins, influencing critical metabolic
511 processes. As well discussed by Passaia and Margis-Pinheiro (2015), GPXs,
512 together with peroxiredoxins, overall can scavenge low endogenous H_2O_2 levels,
513 having an important role as redox sensor due to their highly reactive thiols groups.
514 This could explain the inexistence of differences in ROS accumulation comparing
515 NT and GPX3s. These proteins can oxidize proteins-thiols, and this is an
516 important and specific characteristic because allows oxidation of proteins not
517 directly reactive with peroxide, acting as intermediates that transfer the redox
518 signal by different ways (Klatt and Lamas, 2000; Brigelius-Flohé and Flohé,
519 2011). However, novel approaches are required to unveil the specific GPX3 role
520 in signaling linking respiration and photosynthesis processes. Further
521 experiments are necessary to elucidate the crosstalk between mitochondria and
522 chloroplast to determine the GPX3 role in photosynthetic metabolism during salt
523 stress.

524 5. CONCLUSION

525 Together, our results indicate that mitochondrial Os*GPX3* plays a crucial
526 role in rice protection against salt stress. This is a pioneer study characterizing
527 *OsGPX3* under stress conditions and demonstrates an important role in

528 regulating seed germination, early stages of rice growth and plant defense
529 against salt stress. The deficiency of GPX3 led to a biomass reduction,
530 photosynthetic machinery impairment, alterations in leaf gas exchange and
531 photosynthetic pigment contents under salt stress. The effects of GPX3 silencing
532 on rice photosynthesis during salt stress indicate that redox status can act as a
533 molecular regulator of cross-talk between chloroplasts and mitochondria,
534 suggesting a novel role to this enzyme beyond its role as ROS-scavenger.

535 **Conflicts of interest**

536 The authors have no conflicts of interest to declare.

537 **Contributions**

538 ALSP conducted all experiments, performed biochemical measurements,
539 interpretation and discussion of results and paper writing. GP was co-advisor and
540 designed and obtained the transformed plants. AKL helped in gas-exchange
541 analysis and DJM in biochemical determinations. JAS interpreted results and
542 offered the infra structure for physiology analysis. MMP was the research advisor
543 and helped, together with AKL, with writing and discussion of the manuscript.

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2. CAPÍTULO 2

Este capítulo é referente ao artigo "The OsGPX3 controls ABA response both dependent and independent of H_2O_2 signaling in rice", que será submetido para publicação na revista *Molecular Plant*.

Considerando os resultados do primeiro capítulo, onde a GPX3 parece atuar além dos mecanismos clássicos de remoção de ERO, aplicamos a técnica de proteômica comparando plantas GPX3s e NT para investigar com mais profundidade as rotas e mecanismos regulados pela GPX3. Esse estudo revelou que inúmeras proteínas relacionadas com resposta ao hormônio ácido abscísico foram alteradas e que GPX3 parece estar na rota da cascata de sinalização de ABA, regulando diferentes respostas.

Capítulo II

Papel do gene Os*GPX3* nas vias de resposta do hormônio ácido abscísico em arroz

The mitochondrial Glutathione Peroxidase 3 (*OsGPX3*) regulates ABA responses in rice plants

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ABSTRACT

Oxidative stress is a phenomenon that results in overproduction and accumulation of reactive oxygen species (ROS). Plants developed efficient mechanisms composed by ROS- scavenger enzymes, like glutathione peroxidase (GPX), that allow them to cope with oxidative damages. Besides their role in redox homeostasis, GPXs have also been shown to be involved in signal transduction. To elucidate the pathways regulated by *OsGPX3* we used a proteomic approach comparing RNAi plants for *OsGPX3* (GPX3s) plants and its corresponding non-transformed plants (NT). GPX3s plants displayed altered abundance of proteins involved in abscisic acid (ABA) response and epigenetic processes. RT-qPCR and GUS-staining showed that *OsGPX3* is induced by ABA treatment, suggesting its role in ABA pathway. The analysis of ABA-related responses showed that ABA is unable to inhibit seed germination, inhibit ROS accumulation and inhibit stomata closure in GPX3s plants. GPX3s and NT plants have similar phenotype under drought stress. However, GPX3s were more sensitive to ABA treatment under darkness compared with NT plants. This is the first study demonstrating that *OsGPX3* play a role in ABA signaling and corroborate that redox homeostasis enzymes can act in different and complex pathways in plants cells.

Keywords: *Oryza sativa*; Glutathione Peroxidase; Proteomic; Abscisic acid

34 **1. INTRODUCTION**

35 Plants are continuously submitted to stressful conditions that imply
36 significant damages and productivity losses (Pandey et al., 2017). Oxidative burst
37 is the overproduction and accumulation of reactive oxygen species (ROS) and is
38 activated in response to most stresses (Low and Merida, 1996; Turkan, 2018).
39 The consequences of ROS levels in cells have been extensively studied. Most of
40 the studies discuss an essential role of these molecules as signaling compounds
41 at low levels whereas it is toxic at high levels. However, currently is known that
42 this discussion is too simplistic to represent real cell processes and interrelation
43 between them (Foyer et al., 2017).

44 During a long time, ROS studies were focused in exploring the imbalance
45 in ROS and ROS-scavenger enzymes as a crucial issue to the plant survival
46 under adverse conditions (Birben et al., 2012). However, its currently known that
47 ROS-scavenger enzymes can also induce ROS accumulation and that protein
48 oxidation is not always undesirable, being a crucial signaling step to plant
49 tolerance (Hackenberg et al., 2013; Johnston et al., 2015; Foyer et al., 2017).
50 Therefore, ROS can play numerous roles in plant development and
51 environmental responses. Plant ROS perception is tightly intertwined with
52 signaling cascades through phytohormones (Han et al., 2013; Waszczak et al.,
53 2018). It has been reported that in animal’s diseases, ROS are not the cause but
54 they represent the disease response (Naviaux, 2012). Therefore, explore
55 metabolic conditions that create and regulate them should be targeted for
56 therapy, not only the oxidative changes.

57 ROS-scavenger enzymes with thiol groups, such as the glutathione
58 peroxidase (GPX), can act as redox-sensitive sensor triggering repair
59 mechanisms and regulating plant development and defense responses (Passaia
60 and Margis-Pinheiro, 2015). GPXs are included in non- heme thiol peroxidase
61 class and use mostly glutathione and thioredoxin as a substrate in animal and
62 plants, respectively. These peroxidases catalyze the reduction of hydrogen
63 peroxide (H_2O_2) and others organics compounds to water (Navrot et al., 2006;
64 Margis et al., 2008). Nevertheless, biochemical and enzymatic characterization
65 of GPX has shown that these proteins possess high reactivity toward H_2O_2, but
66 low catalytic efficiency, scavenging low levels of H_2O_2 comparing to other
67 antioxidant enzymes (Fourquet et al., 2008). It seems reasonable to suggest that

68 possible novel functions in addition to redox homeostasis are existent (Passaia
69 and Margis-Pinheiro, 2015). Due to these reasons, these enzymes have emerged
70 as good target to demonstrate that the plant ROS-scavenger enzymes can play
71 a signaling role.

72 Rice (*Oryza sativa* L.) is an important monocotyledon plant and has been
73 extensively used as a model. This crop feeds more than half of the world's
74 population, although its productivity can be affected by different pressures in
75 many areas (Chen et al., 2018). Thus, the identification and characterization of
76 genes involved in the oxidative stress is essential to understand how this fine-
77 tuned regulation occurs and also to provide candidates to plant breeding
78 programs.

79 *In silico* analysis of GPX family in rice was shown that it is composed of
80 five isoforms, ubiquitously distributed in different cell organelles (Margis et al.,
81 2008). The subcellular localization was further confirmed and it was
82 demonstrated that there are two genes encoding mitochondrial proteins
83 (*OsGPX1* and *OsGPX3*), one cytosolic (*OsGPX2*), one chloroplastic (*OsGPX4*)
84 and one cytosolic anchored in the endoplasmic reticulum (*OsGPX5*). Transgenic
85 plants with silenced *OsGPX3* gene (GPX3s) were characterized by shorter roots
86 and shoots and higher mitochondrial H_2O_2 release (Passaia et al., 2013).
87 Although GPX3s plants were also more sensitive to moderate and severe salt
88 stress, showing lower biomass and impairments in many physiological traits, this
89 response didn't seem to be due to higher ROS accumulation (Paiva et al., 2018).
90 Together, these results suggest a novel GPX role regulating stress defense and
91 retrograde signaling between chloroplasts and mitochondria. However,
92 processes modified following GPX3 knockdown are crucial to outline its specific
93 role in plant signaling.

94 Here, by using a proteomic approach, we show that transgenic GPX3s
95 plants do not accumulate many ABA-related proteins, compared with non-
96 transformed plants (NT). ABA exogenous treatment did not induce ubiquitin,
97 actin, and proteins related to vesicle formation in GPX3s, while it induces GPX3
98 gene and promotor expression. Further investigation of ABA-related processes
99 showed that GPX3s display a lower responses to seed germination inhibition,
100 ROS accumulation, and stomata closure compared with NT plants. GPX3s and
101 NT have similar phenotype under drought stress, whereas GPX3s plants were

102 more sensitive to ABA treatment under dark than NT. Taken together, our results
103 provide insights into OsGPX3 role in signaling, being an important component to
104 ABA responses in rice plants.

105 2. RESULTS

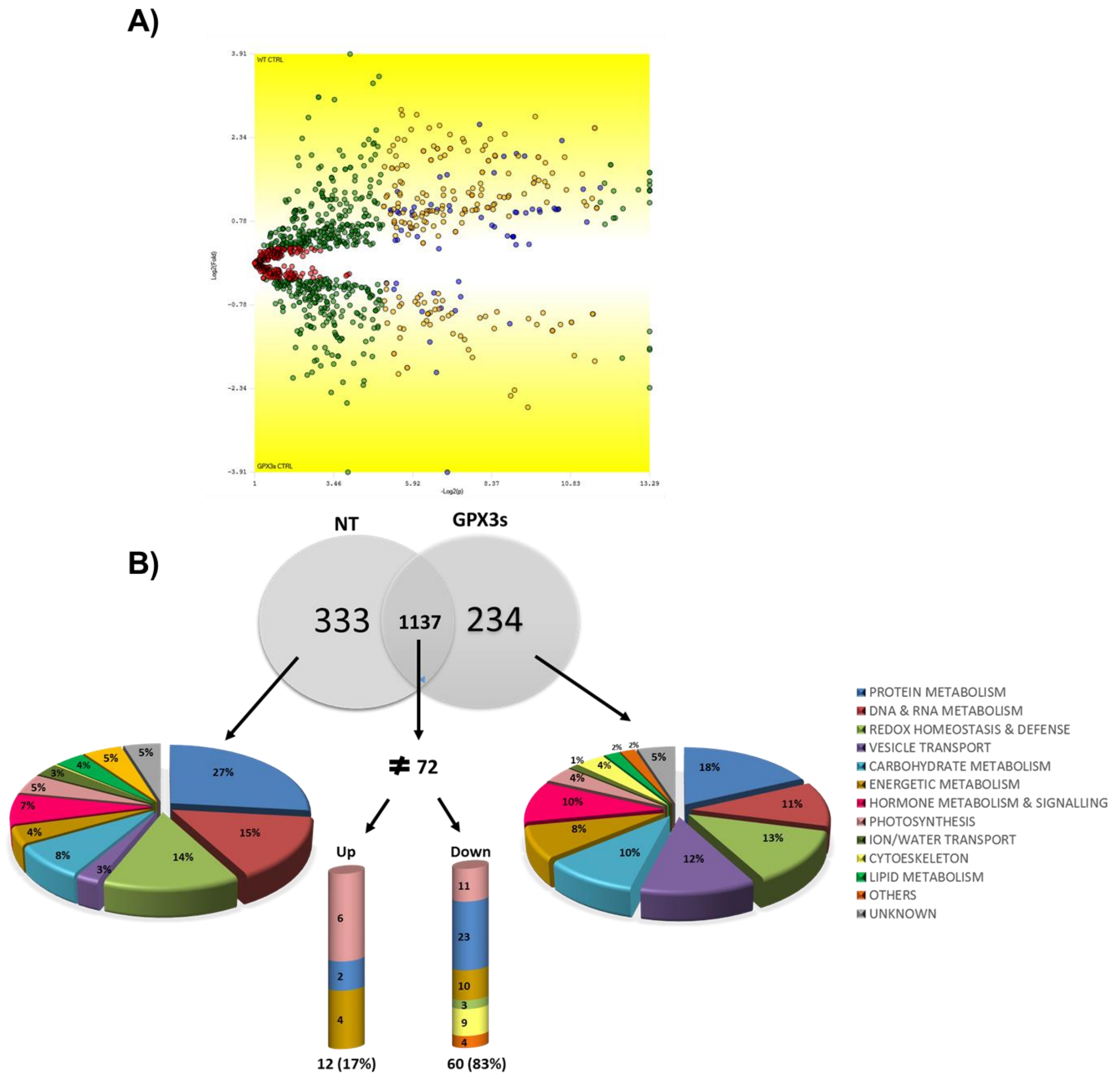
106 2.1 GPX3 silencing induces changes in proteome related to ABA signaling

107 To investigate the alterations induced by *OsGPX3* silencing, a label-free
108 quantitative shotgun proteomic approach using PatternLab for proteomics
109 software (Carvalho et al., 2015) was performed to identify proteins differently
110 identified in GPX3s compared to NT plants under control conditions. First, a T-
111 fold analysis plotted a graph with fold-change versus probability and distributed
112 peptides (represented as dots) in different colors according to statistical results
113 (**Fig. 1A**). It was found 268 red dots that represent proteins that satisfy neither
114 the fold change cutoff nor the FDR cutoff. Green dots (n=585) represent proteins
115 that meet the fold-change cutoff but not FDR cutoff. Orange dots (n=211) are
116 those that satisfy both the fold change cutoff and FDR cutoff but are low abundant
117 proteins and thus require further experimentation to certify their differential
118 expression. Finally, blue dots (=71) are proteins that satisfy all statistical filters
119 and are those that were further selected (Carvalho et al., 2012).

120 Quantitative results showed that 333 and 234 proteins were exclusively
121 identified in NT and GPX3s, respectively. In contrast, 1137 proteins were found
122 in both NT and GPX3s plants, of which 71 were differentially identified: 12 up
123 (17%) and 60 down-regulated (83%) in GPX3S plants compared with NT
124 (**Fig.1B**).

125 Among proteins identified in both NT and GPX3s, many proteins related
126 with ubiquitination and actin were down-regulated in GPX3s **(Table S1**). Among
127 proteins that were found exclusively in NT or GPX3s, it was observed a lower
128 amount of proteins related with protein and DNA/RNA metabolism and redox, as
129 ubiquitin and key proteins in epigenetic processes in GPX3s (**Table S2**). Many
130 prohibitin proteins were also exclusively found in NT. Prohibitins are proteins that
131 play a crucial role in mitochondrial biogenesis and protection against stress and
132 senescence in plant cells. On the other hand, it was observed GPX3s-exclusive
133 proteins related with vesicle transport, cytoskeleton, hormone metabolism,

134 signaling and ion/water transport (**Table S2**). In **Table 1** are listed some selected
135 proteins differentially identified in GPX3s.



136 **Figure 1**. Label-Free quantitative and qualitative results comparing non-
137 transformed (NT) and GPX3s plants under control conditions. **A)** TFold pairwise
138 analysis, where each protein is mapped as a dot on the plot according to its –
139 $\log_2$(P-value) (x-axis) and $\log_2$(fold change) (y-axis). There are 268 red dots that
140 represent proteins that satisfy neither the foldchange cutoff nor the FDR cutoff α.
141 Green dots (n=585) are those that satisfy the fold-change cutoff but not α. Orange
142 dots (n=211) are those that satisfy both the foldchange cutoff and α but are low
143 abundant proteins and thus require further experimentation to certify their
144 differential expression. Blue dots (n=72) are proteins that satisfy all statistical

145 filters. **B)** Venn diagram demonstrating the protein overlap between the NT and
146 GPX3s proteome. There are 333 and 234 unique proteins to NT and GPX3s,
147 respectively. Among the shared proteins (1137), 71 were differentially identified,
148 12 up and 60 down in GPX3s plants, compared with NT. Different colors
149 represent different biological processes, represented in the legend in the right.
150

151 **Table 1**. Selected proteins differentially identified in label-free proteome
152 comparing NT and GPX3s plants under control conditions. The complete lists are
153 in Tables S1-2).

Proteins up-regulated in GPX3s, compared to NT plants			
Uniprot Code	**Fold Change**	**Identity (%)**	**Description**
Hormone Metabolism			
Q6YXT5	GPX3s-Exclusive	100	Putative oxidase-like
Q7XH05	GPX3s-Exclusive	100	Probable aldehyde oxidase 1
P29250	GPX3s-Exclusive	100	Linoleate 9S-lipoxygenase 2
A0A0P0W1T3	GPX3s-Exclusive	100	Lipoxygenase
Q7G794	GPX3s-Exclusive	100	Putative linoleate 9S-lipoxygenase 3
Q53RB0	GPX3s-Exclusive	100	Probable linoleate 9S-lipoxygenase
Q5KQE5	GPX3s-Exclusive	100	Putative S-adenosyl-L-methionine:salicylic acid methyltransferase
A0A0N7KHW1	GPX3s-Exclusive	100	Lipoxygenase
A0A0P0W2N8	GPX3s-Exclusive	100	Lipoxygenase
Q8H4S6	GPX3s-Exclusive	100	Probable protein phosphatase 2C
Q762A8	GPX3s-Exclusive	100	BRI1-KD interacting protein 106
Q762A8	GPX3s-Exclusive	100	BRI1-KD interacting protein
Q7X8B5	GPX3s-Exclusive	100	Calcium-transporting ATPase 5, plasma membrane-type
Q2QMX9	GPX3s-Exclusive	100	Calcium-transporting ATPase 10, plasma membrane-type
Q2QMX9	GPX3s-Exclusive	100	Calcium-transporting ATPase 1, plasma membrane-type
A0A0P0WM50	GPX3s-Exclusive	100	Cation-transporting ATPase (Fragment)
Q7X8B5	GPX3s-Exclusive	100	Calcium-transporting ATPase
Vesicle Transport			
6KA61	GPX3s-Exclusive	100	Putative GTP-binding protein typA
Q6L5I0	GPX3s-Exclusive	95,6	Putative GTPase
Q10LA0	GPX3s-Exclusive	100	Beta-adaptin-like protein
A0A0E0MVI8	GPX3s-Exclusive	100	Protein translocase subunit SecA
Q7EYX7	GPX3s-Exclusive	100	Sec31p
Q6H8D5	GPX3s-Exclusive	100	Coatomer subunit beta'-2
Q6Z0Y9	GPX3s-Exclusive		Sec13p
R7W8L5	GPX3s-Exclusive	86	Vacuolar-sorting receptor 1
Q5JLU1	GPX3s-Exclusive	100	Putative RAB7D
Q75IJ1	GPX3s-Exclusive	100	Putative rab7 protein
Q6ZC54	GPX3s-Exclusive	100	Putative GTP-binding protein Rab7a
A0A0P0VTN1	GPX3s-Exclusive	100	Putative shoot gravitropism 2
A0A1D6PXA1	GPX3s-Exclusive	87,4	Phospholipase SGR2
A0A1D6LLP4	GPX3s-Exclusive	83,6	AP-1 complex subunit gamma-1
Q8H852	GPX3s-Exclusive	100	Coatomer subunit gamma-1
Q6Z382	GPX3s-Exclusive	100	Coatomer subunit gamma-2

B7EEW9	GPX3s-Exclusive	100	Coatomer subunit gamma
Q2R4A0	GPX3s-Exclusive	100	Protein transport protein Sec24-like
Q2R4A0	GPX3s-Exclusive	87	Protein transport protein Sec24-like CEF
A0A1D6PT74	GPX3s-Exclusive	88,7	Protein transport protein Sec24-like
Q53PC7	GPX3s-Exclusive	100	Coatomer subunit beta-1
Q94LY4	GPX3s-Exclusive	100	Sec31p
Q65X08	GPX3s-Exclusive	100	Putative N-ethylmaleimide sensitive fusion
A0A1D6Q5Z6	GPX3s-Exclusive	90,8	Vesicle-fusing ATPase
A0A1E5UNB1	GPX3s-Exclusive	93	Protein TPLATE
A0A1D6N3B3	GPX3s-Exclusive	96,7	AP-4 complex
A0A1D6NA59	GPX3s-Exclusive	82,5	Vps51/Vps67 family
Q94LY4	GPX3s-Exclusive	100	Sec31p
C0PHF1	GPX3s-Exclusive	87,6	AP-4 complex
Ion/Water Transport			
Q6L4R5	GPX3s-Exclusive	100	V-type proton ATPase
Q8GRT8	GPX3s-Exclusive	100	Aquaporin PIP2-4
Q7XUA6	GPX3s-Exclusive	100	Probable aquaporin PIP2-3
Q8GRT8	GPX3s-Exclusive	96,6	Aquaporin PIP2-4
Q7XLR1	GPX3s-Exclusive	100	Probable aquaporin PIP2-6
P50156	GPX3s-Exclusive	100	Probable aquaporin TIP1-1
O80414	GPX3s-Exclusive	100	Mitochondrial phosphate transporter
A0A1E5UMT4	GPX3s-Exclusive	90,8	Mitochondrial phosphate carrier protein 3
Q69TX3	GPX3s-Exclusive	100	Putative mitochondrial phosphate transporter
A0A1D6H1R5	GPX3s-Exclusive	100	V-type proton ATPase A

Proteins down-regulated in GPX3s, compared to NT plants			
Uniprot Code	**Fold Change**	**Identity (%)**	**Description**
Protein Metabolism			
A0A0P0X6U8	1,408463189	100	Putative ubiquitin
Q67V00	1,408463189	100	Putative ubiquitin
Q9ARZ9	1,408463189	100	Ubiquitin-40S
P51431	1,408463189	100	Ubiquitin-40S
A0A0P0X0E0	1,408463189	98	Ubiquitin 11
A0A0P0VF30	1,408463189	100	ubiquitin-like
A0A0P0X005	1,408463189	100	ubiquitin-like
Q58G87	1,408463189	100	Polyubiquitin 3
P0C031	1,408463189	100	Ubiquitin-NEDD8-like RUB2
P0C030	1,408463189	100	Ubiquitin-NEDD8-like RUB1
Q0J9W6	1,408463189	100	ubiquitin-like
P0CH35	1,407751753	100	Ubiquitin-60S
P0CH34	1,407751753	100	Ubiquitin-60S
Cytoskeleton			
Q10AZ4	2,014513586	100	Actin-3 1
Q0IPW3	2,005207297	100	Actin
P0C540	2,001070238	100	Actin-7
A3C6D7	1,940832944	100	Actin-2 1
Q10DV7	1,998541078	100	Actin-1
Q67G20	2,010678953	100	Actin
A0A0P0VAT4	1,916913614	99,5	Actin-like
Q65XH8	1,925662105	100	Putative actin
Q94DL4	1,935861993	100	Putative actin
DNA/RNA metabolism			
Q94JJ7	NT-Exclusive	100	Histone H2B.3
Q7XI22	NT-Exclusive	100	Putative ES43 protein
Q5SMU8	NT-Exclusive	100	RuvB-like helicase
Q6ZBH9	NT-Exclusive	100	Probable protein NAP1
Q6ZBH9	NT-Exclusive	100	Probable protein NAP1

J3KZC5	NT-Exclusive	100	S-adenosylmethionine synthase
Q688F7	NT-Exclusive	99.7	Putative histone deacetylase HD2

154 Considering that several proteins related to ABA synthesis and responses
155 were differentially identified following *OsGPX3*-silencing, as several
156 lipoxygenases, aldehyde oxidase, calcium-transporting ATPase, aquaporins,
157 actins, ubiquitin, cytoskeletal-related and vesicle formation-related (**Table 1**), a
158 label-free proteomic was performed again, comparing NT or GPX3s treated with
159 100 µM ABA during 24 hours, added to the nutrient solution, to investigate if ABA
160 can affect the GPX3s proteome as in NT plants. The TFold pairwise analysis
161 including ABA-treatment is in **Figure S1**.

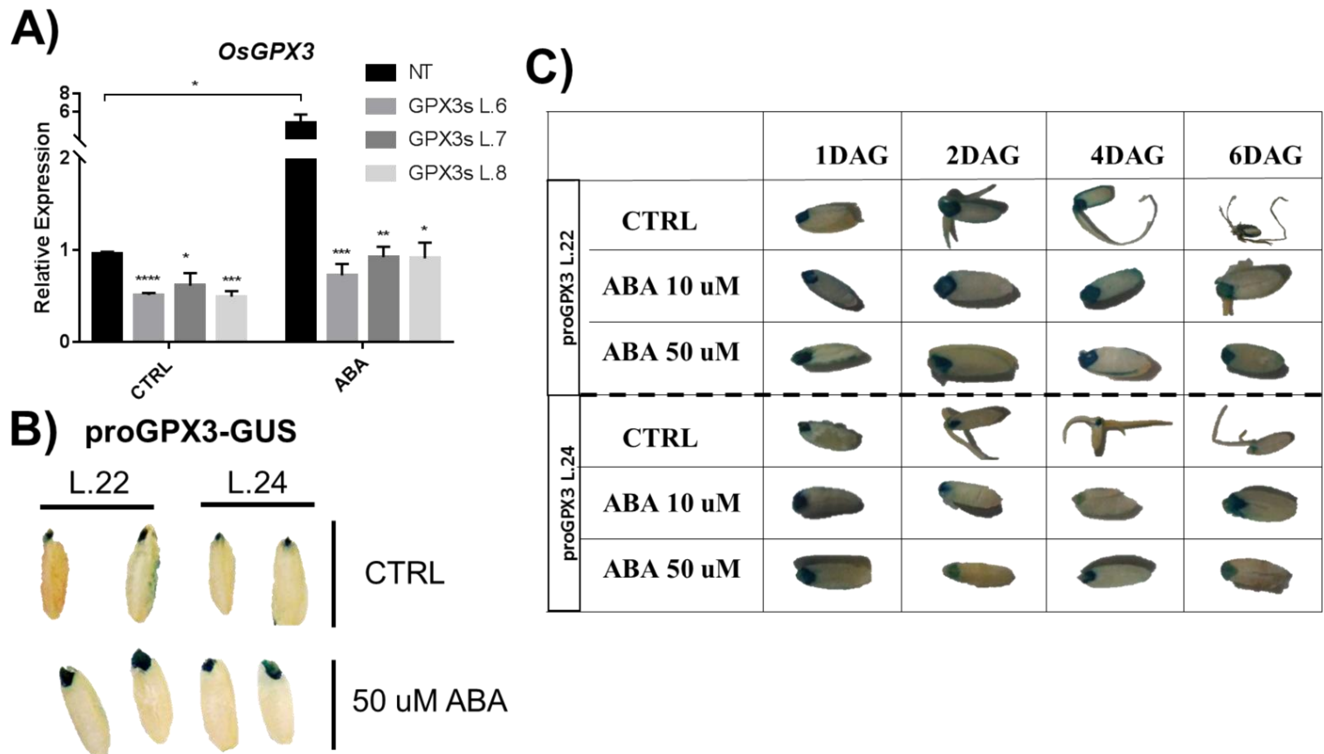
162 The quantitative and qualitative results of comparison between NT under
163 control conditions and after ABA-treatment showed that 312 and 245 proteins
164 were found exclusively in NT under control conditions and after ABA-treatment,
165 respectively, and 1160 proteins were found in both conditions, being 65
166 differentially identified: 58 up (89%) and 7 down (11%) (**Figure S2**). **Table S3**
167 show all proteins differentially identified in NT under control conditions, or 24
168 hours-post-addition of 100 µM ABA in nutrient solution and **Table S4** show
169 proteins exclusively found in NT under control conditions or in NT after ABA
170 treatment. ABA induced the accumulation of many ubiquitin, actin, and vesicle
171 formation-related proteins in NT, as those that were down-regulated in GPX3s
172 compared with NT plants under control conditions.

173 The quantitative and qualitative results of ABA-treatment in GPX3s plants
174 revealed that 246 and 267 proteins were exclusively identified in GPX3s plants
175 under control and after ABA-treatment, respectively, and 56 proteins were found
176 in both conditions, being 54 (96%) up and 2 (4%) down-regulated (**Figure S3**).
177 ABA was able to induce actin accumulation in GPX3s plants but did not induce
178 ubiquitin and proteins related to vesicle formation. Among shared proteins found
179 comparing GPX3s under control conditions or after ABA treatment, plants, only
180 two proteins were found down-regulated in ABA-treated, being two "peroxisomal
181 (S)-2-hydroxy-acid oxidase", responsible for H_2O_2 production (**Table S5**).
182 Interestingly, it was found that many epigenetic-related proteins differentially were
183 regulated. In GPX3s under control conditions it was exclusively found several
184 histones and one "methionine S-methyltransferase", whereas in GPX3s after
185 ABA-treatment it was exclusively found two "methyl-CpG binding domain
186 containing protein" and one "histone-binding protein MSI1", both required to

187 chromatin assembly. **Table S5 and S6** show all proteins differentially identified
188 or exclusively found in GPX3s under control conditions or after ABA-treatment.
189 A direct proteomic comparison between NT and GPX3s after ABA-
190 treatment was performed showing that 249 and 237 proteins were exclusively
191 found in NT ABA-treated or GPX3s ABA-treated, respectively, and 47 proteins
192 were found in both: 9 up (19%) and 38 (81%) down regulated in GPX3s,
193 compared with NT (**Fig. S4).** All proteins differentially identified are listed in **Table**
194 **S7 and S8**. This comparison reinforced that GPX3s ABA-treated plants have
195 lower amount of ubiquitin, actin, two phototropin-2, a probable protein
196 phosphatase 2C and many proteins involved in epigenetic processes (several
197 histones, Histone-lysine N-methyltransferase, Putative ES43 protein, Putative
198 DNA-damage inducible protein, Histone-lysine N-methyltransferase, among
199 others). Altogether, our proteomic results indicated a different regulation of ABA
200 signaling and response in GPX3s plants.
201

202 **2.2 ABA induces GPX3 expression**

203 To investigate the ABA effect in *OsGPX3* gene expression, a leaf RT-
204 qPCR was carried out in NT and GPX3s plants, under control and 6 hours-post
205 treatment with 100 μM ABA (**Fig.2A**). *OsGPX3* gene was induced after ABA
206 treatment, suggesting a role of these gene in ABA route. To confirm that ABA
207 induces *OsGPX3* expression, the expression patterns of the gus gene under the
208 control of the rice GPX3 promoter under control conditions, and after 50 μM ABA
209 treatment were analyzed, using transgenic seed previously generated (Passaia
210 et al., 2013). GPX3 promoter was strongly induced after ABA treatment,
211 reinforcing that ABA induces GPX3 expression (**Fig.2B**). To follow the pattern of
212 GPX3 promoter expression over the time, we analyzed two different lines of the
213 transgenic seeds (proGPX3 L.22 and L.24) 1, 2, 3, 4 and 6 days after germination,
214 under control conditions or with 10 or 50 μM ABA. Our results confirmed that ABA
215 treatment induced GPX3 promotor expression and revealed that GPX3 promoter
216 expression gradually decreases as the germination takes place. On the other
217 hand, ABA inhibit seed germination and keep the GPX3 promoter expression
218 strongly induced, even 6 days after of the beginning of the experiment (**Fig.2C**).
219



220

221 **Figure 2.** Effect of ABA-treatment in *OsGPX3* gene expression. **A)** Shoot RT-
222 qPCR showing the OsGPX3 gene expression comparing non-transformed (NT)
223 and three different lines of GPX3s plants (L.6, L.7 and L.8), under control
224 conditions or 6 hours after 100 µM ABA treatment. NT plants under control
225 conditions were used to normalize transcript accumulation in GPX3s and NT
226 plants. Each bar represents the average of four replicates ± SEM. Asterisks
227 indicate significant differences ($P < 0.05$) comparing with NT in their respective
228 conditions. **B)** Analysis of the expression patterns of two plant lines carrying rice
229 GPX3 promoter and Gus fusion (L.22 and L.24) under control conditions or 24
230 hours-post 50 µM ABA treatment. **C)** Analysis of the GPX3 promoter expression
231 patterns 1, 2, 4 and 6 days after germination (DAG), under control conditions or
232 treated with 10 or 50 µM ABA.

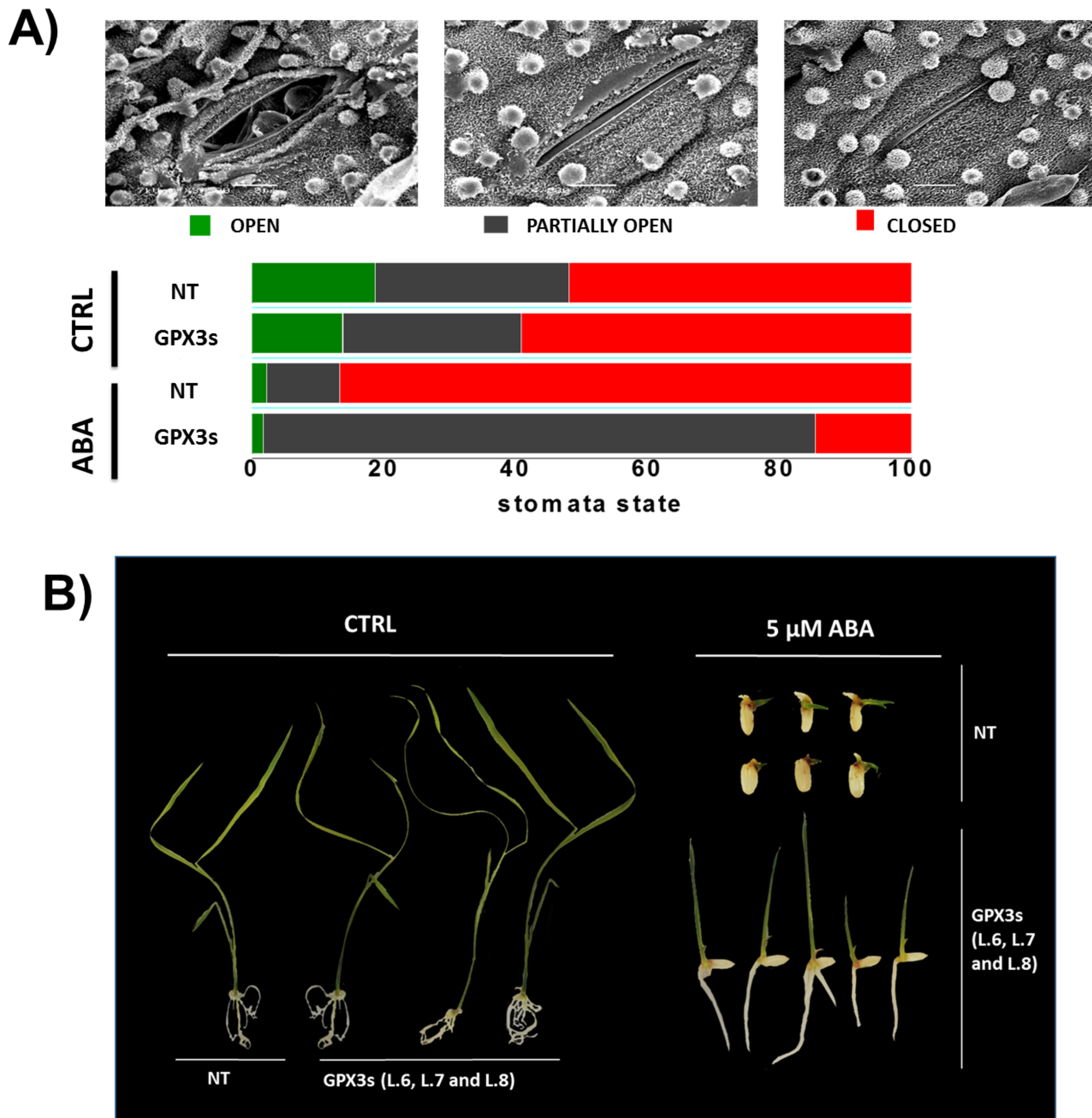
233

234 **2.3 ABA induce different responses in GPX3s**

235 There are several responses directly linked with ABA responses such as
236 stomata closure, seed germination inhibition, ROS accumulation, senescence
237 induction and plant protection against drought stress. Considering that proteomic
238 results revealed that proteins related to ABA responses were differentially

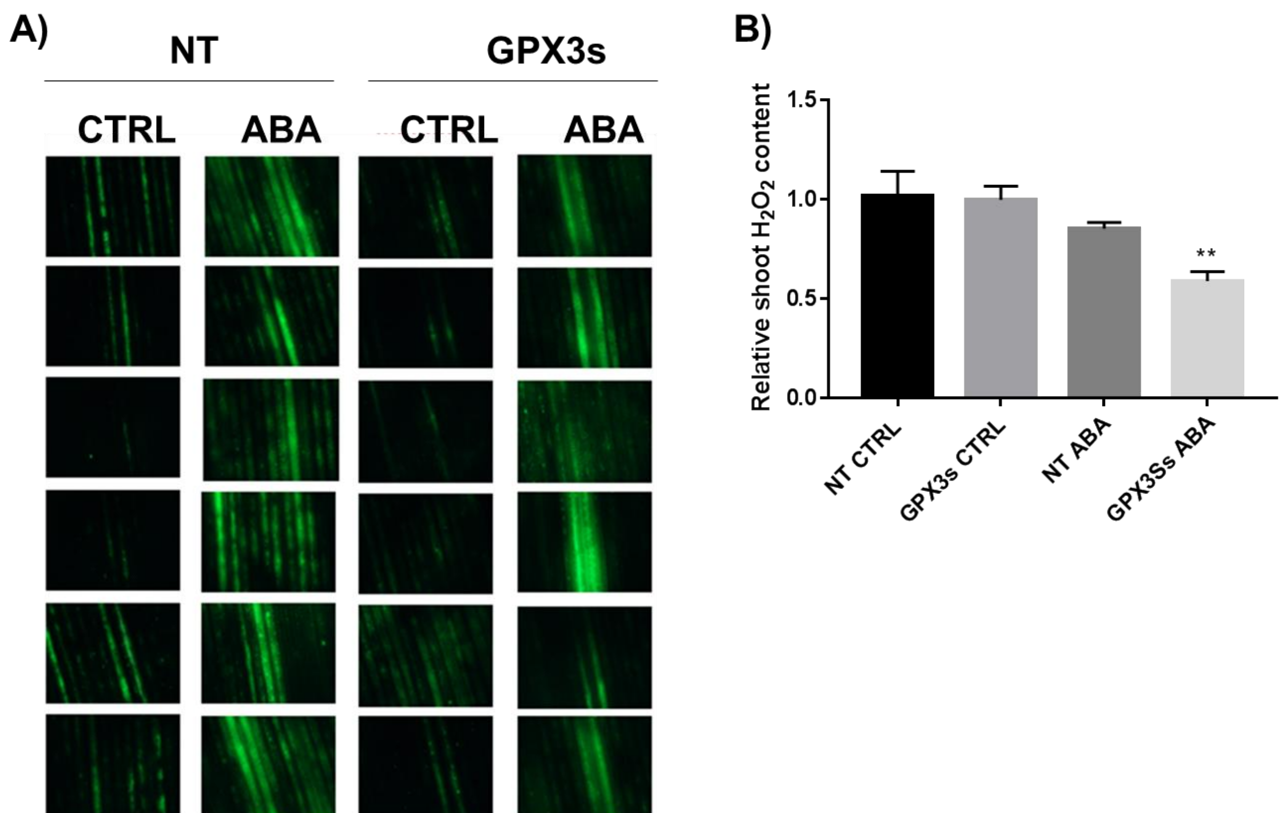
239 regulated in GPX3s, compared with NT plants, we have analyzed how these
240 ABA-related responses are occurring in NT and GPX3s plants.

241 ABA induced stomata closure in NT plants 24 hours after ABA treatment
242 on plant shoots, but did not induce it in GPX3s plants, revealing a significant
243 insensitivity of these plants to ABA (**Fig. 3A)**. The seed germination inhibition
244 assay was done using three different lines of GPX3s (L6., L7 and L8) and results
245 showed that the supplement of 5μM ABA in the nutritive medium completely
246 inhibit the germination of the NT seeds, but not of GPX3s plants, reinforcing a
247 relative insensitivity of these plants to ABA treatment (**Fig. 3B**



248 **Figure 3**. Comparison of ABA effect in non-transformed (NT) and GPX3s plants.
249 **A)** Effect of ABA-treatment on stomata closure, comparing NT and GPX3s under
250 control conditions and 24 hours after 100 µM ABA spray in plant shoots. Three
251 biological replicates were analyzed, using 4 leaf segments of each. The first 200
252 stomata found in each leave segment were classified in three categories, as
253 shown in figure: open, partially open and closed. **B)** Effect of ABA-treatment on
254 seed germination, comparing NT and GPX3s (L.6, L.7 and L.8) 14 days-post
255 germination under control conditions or with supplementation of 5 µM ABA in the
256 medium.

257 The ABA role in ROS induction is also well established. We have analyzed
258 ROS induction 24 hours after sprayed out 100µM ABA on plant shoots. The
259 H_2DCFDA staining showed that ABA-induced ROS accumulation in both plants,
260 but in a lower amount in GPX3s (**Fig 4A)**. The quantitative analysis using Amplex-
261 Red assay, that measure H_2O_2 content in the samples, showed that there were
262 no statistical differences between NT and GPX3s under control conditions or
263 between NT and NT ABA-treated. However, a lower H_2O_2 amount in GPX3s
264 plants after ABA treatment were observed (**Fig 4B)**. This indicates that ABA
265 induces general ROS accumulation in NT plants, but not of H_2O_2. In addition,
266 ABA induced a reduction of H_2O_2 content in GPX3s plants that, consequently,
267 have a lower amount of general ROS induced by ABA. These results confirm that
268 ABA regulates ROS production in NT and GPX3s differentially.

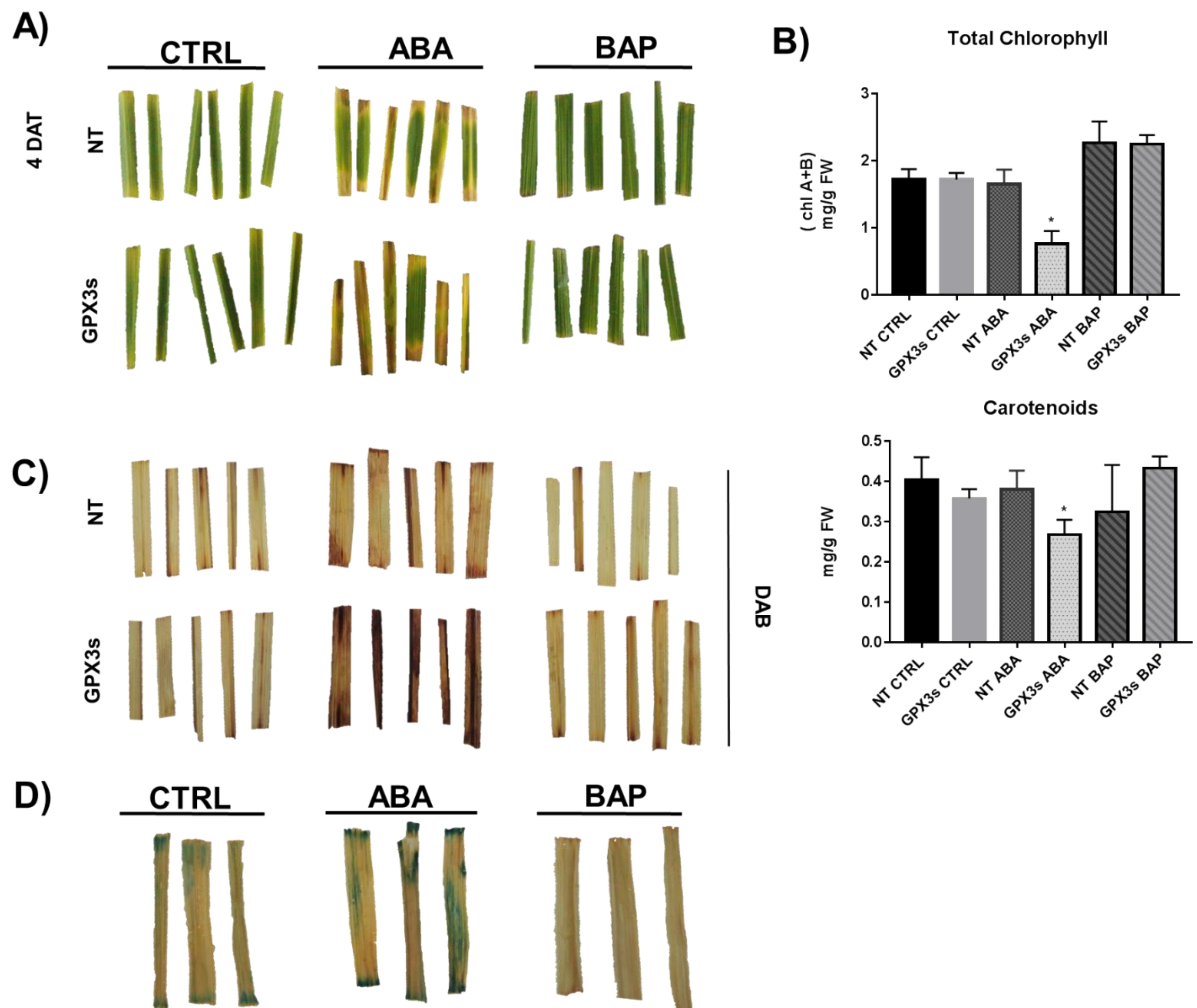


269

270 **Figure 4**. ABA effect on ROS accumulation. **A)** Reactive oxygen species content
271 using 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) staining, comparing
272 non-transformed (NT) and GPX3s plants, under control conditions and 24 hours-
273 after 100 µM ABA sprayed in shoot plants. **B)** H_2O_2 contents in NT and GPX3s
274 plants under control conditions and 24 hours-after 100 µM ABA treatment. Each

275 bar represents the average of six replicates ± SEM. Asterisks indicate significant
276 differences at $p < 0.05$ comparing with NT in their respective conditions.

277 Given that ABA also induces plant senescence under dark conditions
278 (Liebsch and Keech, 2016), the responses of leaf segments in control conditions
279 (H_2O) or in the presence of 100µM ABA or 100µM BAP were analyzed under
280 darkness. ABA induced plants senescence in both, NT and GPX3s plants, but
281 this effect was more intense in GPX3s plants (**Fig 5A**). To confirm that these
282 plants were really more affected than NT, pigment contents were measured,
283 confirming that GPX3s plants had a lower amount of total chlorophyll (a+b) and
284 carotenoids four days after ABA treatment (**Fig. 5B**). To evaluate if this response
285 was directly related to ROS accumulation, we also verified H_2O_2 accumulation
286 using DAB staining confirming that leaf segments treated with ABA under dark
287 conditions accumulated H_2O_2. This accumulation was more pronounced in
288 GPX3s than NT, corroborating our previous analyzes (**Fig. 5C**). To confirm that
289 *OsGPX3* gene has an important role in this specific response, we submitted the
290 GPX3-promoter-GUS plants to the same conditions to analyze the promoter
291 expression. GPX3 promoter was strongly induced by ABA and repressed by BAP
292 (**Fig. 5D)**. Together these results revealed that GPX3s plants seem to be more
293 sensitive to ABA effect related to senescence, having a lower amount of pigment
294 contents and higher accumulation of ROS in these conditions. Furthermore,
295 *OsGPX3* has an important role in this response, given that its promoter was
296 induced, explaining the sensitivity of GPX3s plants to these conditions.



297 **Figure 5**. Detached leaves response to dark-induced senescence. **A)**
298 Senescence response in non-transformed (NT) and GPX3s plants under control
299 conditions (H_2O) or 4 days after treatment with 100 µM of ABA or BAP. **B)** Total
300 chlorophyll (a+b) and carotenoid contents. Each bar represents the average of
301 four replicates ± SEM. Asterisks indicate significant differences at $p < 0.05$
302 comparing with NT in their respective conditions. **C)** H_2O_2 accumulation using
303 3,3′-Diaminobenzidine (DAB) staining. **D)** Analysis of the GPX3 promoter
304 expression patterns.

305

306 Finally, considering that ABA induces plant protection to drought stress
307 (Vishwakarma et al., 2017) we submitted 40-days-old NT and GPX3s plants to
308 drought stress to evaluate their response to water privation and also if ABA can
309 protect both against the stress. The treatment with 100µM ABA sprayed on plant

310 shoots induced plant protection against drought stress. However, there were no
311 visual differences between NT and GPX3s plants (**Fig. S5**). Thus, the differential
312 regulation of ABA signaling in NT and GPX3s plants was not enough for plants
313 to show phenotypical differences in response to drought T this specific
314 developmental stage.

315 To verify if the differences observed in NT and GPX3s occurred also at
316 transcript level, we evaluated the expression levels of some gene targets as
317 ubiquitin and genes related with ABA synthesis (NCED3), catabolism (Oxidase3)
318 and signaling (Abi5 and DREB2A), under control conditions and 6 hours after
319 ABA-treatment, applied in the same conditions of proteomic experiments,
320 comparing NT and three different lines of GPX3s plants (L.6, L.7, and L.8).
321 Results showed that ABA treatment induced ubiquitin expression in NT plants,
322 but not in GPX3s, as revealed in the proteomic analysis (**Fig. S6)**. There were no
323 statistical differences in the expression of gene *OsNCED3* in control conditions.
324 ABA-treatment induced NCED expression in NT plants, but in GPX3s it was
325 repressed. The *OsAbi5* gene, a basic leucine zipper transcription factor that plays
326 a key role in the regulation of ABA responses, was up-regulated in GPX3s lines
327 under control conditions. After ABA treatment, however, *OsAbi5* was repressed
328 in GPX3s, compared with NT, while NT induced its expression, in comparison
329 with control conditions. The *OsDREB2A* is a dehydration-responsive element
330 binding transcription factors and was strongly induced after ABA treatment in NT
331 plants. In turn, GPX3s was not able to induce it. The ABA-catabolic gene
332 *OsABA8ox3* was also down-regulated in GPX3s plants, in both conditions,
333 control, and after ABA treatment. NT, in turn, induced it after ABA-treatment (**Fig.
334 S6)** Together, these results showed NT and GPX3s have differences in the
335 expression of key genes in the ABA pathway. GPX3s cannot induce expression
336 of ubiquitin, OsAbi5, OsDREB2A, and OsABA8ox3, crucial to ABA responses.

337

338 **2.4 GPX3 silencing induces signal transducer glutathionylation**

339 To understand how the *OsGPX3* silencing can affect the ABA-pathway
340 directly, we hypothesized that the glutathione peroxidase knockdown could
341 induce an imbalance in post-translational modifications, as glutathionylation
342 rates. To verify it, the raw files generated in label-free proteomic described before
343 were used again, including a new variable modification to S-glutathionylation

344 (Delta mass: +305.0681) in search comet parameters. This analysis allowed us
345 to find targets that have that specific modification, although the protein extraction
346 protocol was not made to enrich the samples with cys-proteins.

347 The search revealed that one protein, whose Uniprot code is “Q6Z8S7”,
348 was S-glutathionylated and accumulated in response to the *OsGPX3*-silencing.
349 This protein is described as a “putative signal transducer and activator of
350 transcription interacting protein” and acts in histone acetylation and DNA
351 methylation/demethylation. This finding suggests that *OsGPX3*-silencing can
352 result in post-translational modifications, activating or repressing protein function.

353

354 3. DISCUSSION

355 The classical discussion that ROS always acts as negative molecules at
356 high concentrations and that the main role of antioxidant enzymes is as ROS-
357 scavenger have been gradually changing by works that are revealing the great
358 complexity of these interactions (Foyer et al., 2017). Although the responses to
359 oxidative stresses have been extensively studied, how activated responses are
360 switched “off” or “on” when oxidative stress is induced remain poorly investigated.

361 We have previously showed that although GPX3s plants were more
362 sensitive to salt stress, this is not due to higher ROS accumulation, suggesting a
363 novel role for GPX3 beyond its classical antioxidant function (PAIVA et al., 2018).
364 Here we further describe that *OsGPX3*-silencing disturbs the epigenetic
365 regulation in rice plants, having a lower accumulation of histones, histone
366 deacetylases, key proteins in DNA methylation/demethylation and in chromatin
367 assembly and remodeling.

368 The epigenetic regulation of the genes involved in oxidative stress
369 response is largely unknown but has emerged as a hot topic (Grek et al., 2013;
370 Kumar, 2018; Zappe et al., 2018). Here we showed that the *OsGPX3*-silencing
371 induced the S-glutathionylation of a signal transducer that acts controlling the
372 histone code. This discovery is strongly supported by other reports that showed
373 that a glutathione peroxidase knockdown can directly affect the glutathione rate
374 available in the plant cell (Noctor et al., 2012) and that the maintenance of
375 glutathione levels is very important to epigenetic regulation (García-Giménez et
376 al., 2014). An α-ketoglutarate dehydrogenase, for example, was reversibly
377 inactivated by S-glutathionylation in response to alterations in the mitochondrial

378 GSH status (Nulton-Persson et al., 2003) and the histone 3 was S-
379 glutathionylated in mammalian cells and tissues, describing glutathione as new
380 post-translational modifier of the histone code that alters the nucleosome
381 structure (García-Giménez et al., 2017). S-glutathionylation roles are associated
382 with specific protein inactivation or protection against protein damage induced by
383 higher levels of oxidative stress, being a way to protect sensitive cysteinyl
384 residues from irreversible oxidation forms (Dixon et al., 2005; Dalle-donne et al.,
385 2007). Thus, *OsGPX3*-silencing did not induce enough oxidative stress to be
386 detected by standard methodologies, but was enough to induce alterations in
387 chromatin proteins and, consequently, to activate or repress specific genes.

388 In Arabidopsis, it was already shown that the *PQT3* gene, which encode
389 an E3 ligase protein, was able to interact directly with a methyltransferase protein
390 (PRMT4b). This interaction catalyzes histone methylation on APX1 and GPX1
391 chromatin and induces their expression, protecting plants against oxidative
392 stress. In turn, PQT3 levels increase when oxidative stress declined and acts as
393 E3 ubiquitin ligase to specifically target PRMT4b for degradation (Luo et al.,
394 2016). This example highlights the importance of histone methylation in gene
395 activation and the importance of ubiquitination in the control of protein availability.

396 GPX3s plants has a lower amount of ubiquitin protein, and the regulation
397 of ubiquitin enzymes by glutathione following oxidative stress was already
398 reported (Jahngen-hodge et al., 1997). Here we showed that ABA induces
399 ubiquitin accumulation in NT plants, but not in GPX3s. Ubiquitination is crucial to
400 control several processes in plant cell as development, protein-turnover, stress
401 defense, and hormone signaling pathways and can alter protein assembly,
402 localization, activity, and interaction ability (Kelley, 2018). A crucial ubiquitin role
403 in ABA-pathway is well documented. The ABA signaling pathway is composed of
404 phosphatases, kinases, transcription factors, and membrane ion channels and
405 different types of E3 ligases can mediate ubiquitination of all these structures (Yu
406 et al., 2016; Yang et al., 2017). Besides that, ubiquitin and the proteasome
407 system are known targets of S-glutathionylation due to the presence of cysteine
408 residues located in the α-ring (Demasi et al., 2014). Thus, the lower ubiquitin
409 amount in GPX3s plants could be directly regulated by oxidative stress in
410 mitochondrial organelles and glutathione imbalance or indirectly by epigenetic or
411 post-translational modifications.

412 The vast majority of proteins that were down regulated in GPX3s under
413 control conditions were also induced by ABA in NT plants, suggesting that GPX3
414 may play a key role in regulatory mechanisms associated with activation of ABA-
415 responses. The reduction in ubiquitin and actin content could be crucial to GPX3s
416 plants be not able to respond to ABA treatment.

417 Recently, increasing evidence demonstrates that endomembrane
418 trafficking, including endosomal trafficking and the autophagy pathway, controls
419 key regulators turnover, playing vital roles in ABA perception, signaling, and
420 action, regulating the protein compartmentalization and abundance within cells
421 (Yu and Xie, 2017). However, actin filaments play a critical role in vacuolar
422 trafficking at the Golgi complex in plant cells (Kim et al., 2005) and were down-
423 regulated in GPX3s.

424 Taken together, our results show that some protein classes positively
425 related with ABA were up regulated in GPX3s, as vesicle and ion/water transport,
426 but others as ubiquitin and actin were repressed. The complexity between GPX3
427 and ABA signaling indicate that GPX3s are less sensitivity to ABA responses, as
428 stomata closure, seed germination inhibition, and ROS induction, but more
429 sensitive to other conditions, such as in dark-induced senescence, or be similar
430 to NT, as in drought stress response.

431 In **Figure 6** we propose a hypothetical model explaining how cross-talk
432 between *OsGPX3* and ABA might be occur following our proteomic, gene
433 expression and functional experiments, we hypothesize that GPX3 affects ABA
434 signaling by two different ways, namely H_2O_2 dependent and independent
435 pathways. The first one is related to the senescence induced by ABA, and our
436 results indicated that ABA- induced GPX3, which in turn induces prohibitins, that
437 are important enzymes to mitochondrial biogenesis and to plant protection
438 against senescence (Chen et al., 2005; Ahn et al., 2006). Furthermore, GPX3
439 can also act as a ROS-scavenger enzyme, helping the plant to cope with
440 senescence. Together, these results may explain why GPX3s plants were more
441 sensitive to dark-induced senescence, accumulating more H_2O_2 and having lower
442 pigment amount. By contrast, the GPX3 interaction with ABA in an H_2O_2-
443 independent via happens because GPX3 is not acting removing ROS, but in a
444 signaling cascade. We found that GPX3 represses the accumulation and the
445 post-translational modification of S-glutathionylation in a signal transducer that

446 act in epigenetic regulation. Moreover, GPX induces histones, proteins involved
447 DNA methylation/demethylation, ubiquitin, actin, and vesicle formation, that are
448 known as important to ABA responses. GPX3 also induce phototropin-2, that are
449 crucial to stomata movement, and ABA-signaling genes, as *OsDREB2A*, *OsAbi5,*
450 and *OsABA8ox3*. Finally, our proteomic results also showed that GPX3
451 represses a negative regulator of ABA-pathway, a phosphatase C-type (PP2C).
452 Together, these results are in good agreement with GPX3s plants to be more
453 insensitive than NT in certain classical ABA-responses, as stomata closure and
454 seed germination inhibition.
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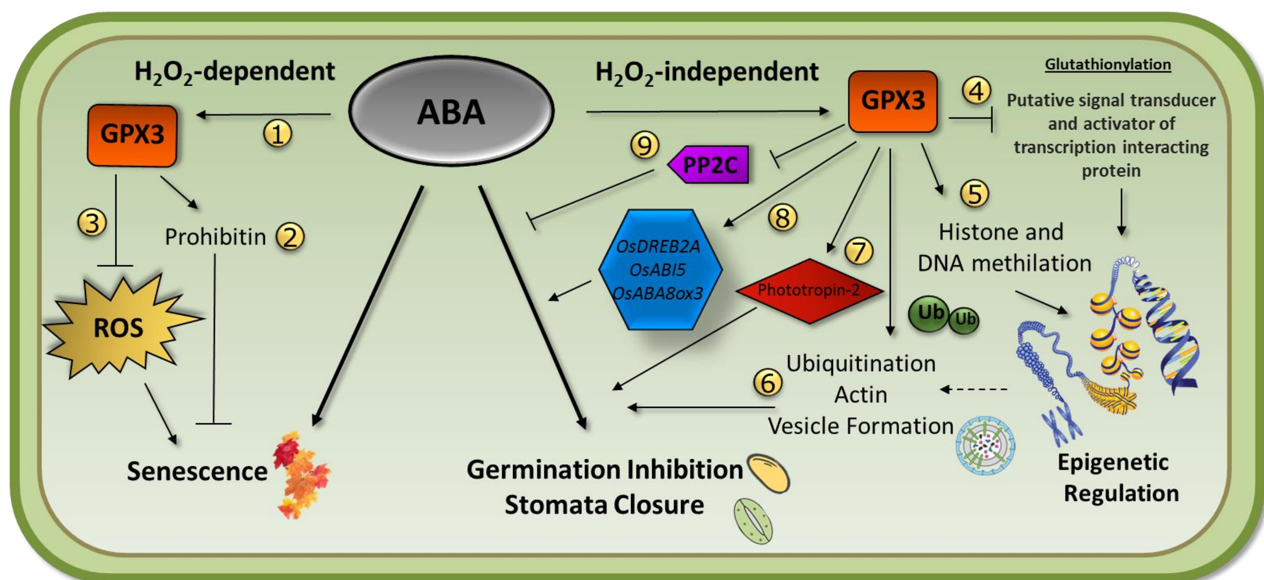

456
457 **Figure 6**. Hypothetical model representing the OsGPX3 role on ABA signaling
458 and responses in rice plants. **1)** ABA induces GPX3 expression. **2)** GPX3 induces
459 accumulation of prohibitin protein, that inhibits senescence and **3)** acts as a ROS-
460 scavenger, also avoiding senescence in rice. **4)** GPX3 repress and prevent the
461 glutathionylation of a signal transducer that act on DNA
462 methylation/demethylation and histone acetylation. **5)** GPX3 induces histones
463 and proteins responsible for DNA methylation, acting directly in epigenetic
464 regulation. **6)** GPX3 also induces accumulation of ubiquitin, actin and diverse
465 proteins related with vesicle formation after ABA treatment, which is crucial for
466 some ABA responses. **7)** ABA treatment induces in GPX3 accumulation of
467 phototropin-2 and **8)** OsDREB2A, OsAbi5, and OsABA8ox3, involved in ABA-

468 signaling. **9)** GPX3 repress accumulation of PP2C protein, which is a negative
469 regulator of ABA signaling.

470 Other examples were recently published showing important antioxidant
471 enzymes, as peroxiredoxins (Prxs) and glutathione peroxidase, acting in
472 signaling cascades (Passaia and Margis-Pinheiro, 2015; Foyer et al., 2017).
473 These enzymes are strong candidates for the sensing mechanism in redox
474 regulation because they contain highly reactive thiol groups proteins (Brigelius-
475 flohe and Flohe, 2011). H_2O_2 has strong reactivity for cysteines, making GPXs
476 and peroxiredoxins ideal enzymes for sensing and signaling, playing biological
477 functions besides those of simple peroxide-protective enzymes (Fourquet et al.,
478 2008). Moreover, the presence of biochemical and enzymatic attributes as high
479 reactivity for peroxide, their relative overall catalytic inefficiency and their the
480 ability to reversibly inactivate substrates contribute in their performance in
481 signaling. In *Saccharomyces cerevisiae*, Gpx3 interacts directly with GAPDH and
482 protects it from NO stress and thereby helps to the maintenance of homeostasis
483 during exposure to NO stress (Lee et al., 2011). In Arabidopsis, GPX3 functions
484 as both a redox transducer and a scavenger in abscisic acid and drought stress
485 responses (Miao et al., 2006). Similarly, *atgpx3* mutation disrupted the activation
486 of calcium channels and the expression of ABA- and stress-responsive genes.
487 Besides that, AtGPX3 can interact with a PP2C and ABI1, being capable of
488 oxidizing the molecules depending on its redox status. Thus, there is increasing
489 evidence highlighting the importance of several redox signaling mechanisms in
490 the regulation of a plethora of cellular processes. In this context, the redox
491 proteome emerges as a useful tool to identify oxidized proteins and to determine
492 the extent and location of oxidative modifications in the proteomes of interest
493 (Butterfield and Perluigi, 2017). This technique helps to elucidate the changes
494 induced in cell redox status and understand how different antioxidant enzymes
495 can also control signaling processes, beyond protect the cell against the negative
496 ROS-induced damage.

497 In conclusion, we have identified a novel component of ABA regulatory
498 pathway involving the mitochondrial isoform glutathione peroxidase 3, that
499 controls epigenetic and ABA responses in rice plants. This regulatory pathway is
500 very complex and far from being completely elucidated, but highlights a full range
501 of possibilities where these enzymes can act. This work provided much

502 information, as protein candidates that could be more explored and be useful for
503 future investigation to understand better the cross-signaling between *OsGPX3*
504 and ABA and how *OsGPX3* can control cell signaling by changing redox status.

505 **METHODS**

506 **Plant growth and treatments to proteomic approach**

507 Rice plants (*Oryza sativa* L. ssp. Japonica cv. Nipponbare) were used in this
508 study. The OsGPX3-silenced rice plants (GPX3s) were previously generated
509 (Passaia et al., 2013). Seeds from non-transformed (NT) plants and GPX3s were
510 germinated in filter paper wet with H_2O at 28 °C in the light, with a 12-h
511 photoperiod. Twelve-days-old seedlings were transferred to plastic pots
512 containing Hoagland's solution (Hoagland and Arnon, 1950) and cultivated in a
513 growth chamber at 28 ± 2 °C, with a 12-h photoperiod. A set of homogenous
514 plants was distributed randomly and divided into two groups (control and ABA-
515 treated). ABA (Sigma-Aldrich) was added in the nutrient solution in the final
516 concentration of 100 µM. The shoot tissues were collected 24 hours post-ABA
517 treatment for proteomics analyzes and after 6 hours to access transcript level
518 using RT-qPCR.

519 **Protein Precipitation and Trypsin Digestion**

520 Protein extraction was performed as previously described by Neilson et al.,
521 (2014) with modifications. Two hundred milligrams of control or ABA-treated leaf
522 material were ground to powder in liquid nitrogen and 2 mL of extraction buffer (8
523 M urea, 100 mM Tris–HCl, pH 8.5, and 1 % SDS) was added for homogenization
524 and was subjected to probe sonication on water for 3 × 20-s pulses. After
525 centrifugation at 17,000 × g, 10 min, 4 ◦C, the supernatant was submitted to
526 protein precipitation adding cold 100% trichloroacetic acid (TCA) in a fresh tube
527 to a final concentration of 25% (v/v). The solution was briefly vortexed, and
528 proteins were precipitated overnight at −20 °C. After centrifugation at 17,000 × g,
529 10 min, 4 ◦C, the supernatant was discarded, and the precipitate washed (3x)
530 with 850 µL of cold acetone and centrifuged as above. The protein pellet was air
531 dried until the acetone evaporated and was solubilized in 7 M urea, 2 M thiourea.
532 An aliquot was used to determine protein concentration by the Qubit Protein
533 Assay Kit (Qubit® 2.0 Fluorometer, Thermo Scientific) according to the

manufacturer's instructions. For protein digestion, 50 μg of proteins of each sample was reduced with dithiothreitol at a final concentration of 10 mM for 1 h at 30 °C, followed by iodoacetamide alkylation at 40 mM final concentration for 30 min at room temperature in the dark. Samples were diluted with 50 mM ammonium bicarbonate to 1 M urea concentration and after trypsin addition (1:50, w/w, Sequencing Grade Modified Trypsin, V5111, Promega), samples were incubated at 35 °C for 18 h. Tryptic hydrolysis was stopped with TFA at 0.1% final concentration. After digestion peptides were concentrated and desalted by custom-made chromatographic Poros 50 R2 (PerSeptive Biosystems) reverse phase tip-columns and dried on vacuum concentrator (Thermo Scientific) (Gobom, 1999).

nLC-MS Analysis

Peptides resuspended in 0.1% formic acid were quantified by the Qubit Protein Assay Kit. MS analysis was performed in triplicates for each biological replicate from NT and GPX3s, in control and ABA-treated conditions, and EFN in a nano-LC EASY-II coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). Two μg of peptides were loaded in a precolumn (2 cm length, 100 μm I.D., packed in-house with ReproSil-Pur C18-AQ 5 μm resin–Dr. Maisch GmbH HPLC) and fractionated in a New Objective PicoFrit® Column (25 cm length, 75 μm I.D., packed in-house with ReproSil-Pur C18-AQ 3 μm resin–Dr. Maisch GmbH HPLC). Peptides were eluted using a gradient from 95% phase A (0.1% formic acid, 5% acetonitrile) to 40% phase B (0.1% formic acid, 95% acetonitrile) for 107 min, 40–95% phase B for 5 min and 95% B for 8 min (total of 120 min at a flow rate of 200 nL/min). After each run, the column was washed with phase B and re-equilibrated with phase A. m/z spectra were acquired in a positive mode applying data-dependent automatic MS and MS/MS acquisition. MS scans (m/z 350–2,000) in the Orbitrap mass analyzer at resolution 30,000 (at m/z 400), 1 × 106 AGC and 500 ms maximum ion injection time, were followed by HCD MS/MS of the 10 most intense multiply charged ions in the Orbitrap at 10,000 signal threshold, resolution 7,500 (at m/z 400), 50,000 AGC, 300 ms maximum ion injection time, m/z 2.5 isolation width, 10 ms activation time at 30 normalized collision energy and dynamic exclusion enabled for 30 s with a repeat count of 1.

566

567 **Database Search and peptide-spectrum matching (PSM)**

568 Bioinformatic analyses were guided by the steps described in the PatternLab
569 for proteomics v.4.0 (PL40) protocol (Carvalho et al., 2015). This software is
570 freely available at http://www.patternlabforproteomics.org. The *Oryza sativa*
571 database was downloaded from Uniprot database February 2017 and a target-
572 decoy database was generated using PatternLab to include a reversed version
573 of each sequence found in the database plus those from 127 common mass
574 spectrometry contaminants. The Comet search engine (Eng et al., 2013) found
575 in PatternLab (4.0.0.84) was used for searching spectra against Uniprot's
576 database. The searches were performed with the following parameters: trypsin
577 digestion with two missed cleavage allowed, accepted semi-tryptic peptide
578 candidates within a 40-ppm tolerance from the measured precursor m/z,
579 considered fixed carbamidomethyl modification of cysteine and variable
580 modification of oxidized methionine and S-glutathionylation cysteine.

581 The Search Engine Processor (SEPro), built into PatternLab 4.0, was used for
582 converging to a list of identifications with < 1% of false discovery rate (FDR) at
583 the protein level, as previously described (Carvalho et al., 2012). Briefly, the
584 identifications were grouped by charge state (2 + and ≥ 3 +), and then by tryptic
585 status, resulting in four distinct subgroups. For each group, the XCorr, DeltaCN,
586 DeltaPPM, and Peaks Matched values were used to generate a Bayesian
587 discriminator. The identifications were sorted in non-decreasing order according
588 to the discriminator score. A cutoff score was established to accept a false-
589 discovery rate (FDR) of 1% at the peptide level based on the number of labeled
590 decoys. This procedure was independently performed on each data subset,
591 resulting in an FDR that was independent of charge state or tryptic status.
592 Additionally, a minimum sequence length of six amino-acid residues was
593 required. Results were post-processed to only accept peptide spectrum match
594 (PSMs) with < 15 ppm from the global identification average. One-hit wonders
595 (i.e., proteins identified with only one mass spectrum) with the peptide having an
596 XCorr of < 1.8 were discarded. This last filter led to FDRs, now at the protein
597 level, to be lower than 1% for all search results.

598

599 **Relative quantitation of proteins and data analysis**

600 To determine the relative abundance of proteins, XIC analysis was used in a
601 label-free relative quantification approach. Proteins were grouped by maximum
602 parsimony and the presence of proteins in at least two out of three replicates was
603 required for each condition. Venn's diagrams were generated from the output of
604 PatternLab's Birds Eye view report. The differential expression proteins were
605 identified and Volcano plots were generated by a pairwise comparison using
606 PatternLab with TFold module between two samples, which uses a theoretical
607 FDR estimator to maximize identifications satisfying both a fold-change cut-off
608 that varies with the *t*-test *p*-value as a power law and a stringency criterion that
609 aims to fish out proteins of low abundance that are likely to have had their
610 quantitation compromised. Only proteins satisfying fold change and p-value
611 criteria were considered as differentially expressed. In the differential protein
612 expression analysis, counting of differently expressed proteins includes all the
613 proteins that have been identified as differentially expressed proteins in either
614 one pair of analysis. Proteins identified with the database description unclear or
615 as "putative uncharacterized protein" were submitted to manual Blastp in Uniprot
616 (http://www.uniprot.org/blast/) and NCBI (https://blast.ncbi.nlm.nih.gov) websites.
617 Proteins with high identity were selected for the identification of uncharacterized
618 proteins.

619 **Data availability**

620 The mass spectrometry proteomics raw data have been deposited to the
621 ProteomeXchange Consortium via the PRIDE partner repository with the dataset
622 identifier (XXXXXX)

623 **Analysis of the rice Gpx3 promoter expression pattern after ABA treatment**

624 Transgenic plants harboring the fusion GPX3 promoter-Gus (promGPX3-GUS)
625 were previously generated (Passaia et al. (2013). Seeds were used to assess the
626 expression of the Gus gene under the control of the GPX3 promoter in control or
627 ABA treatment conditions. Seeds of two different lineages (L. 22 and L. 24) were
628 longitudinally sliced and treated with H_2O or 100 µM ABA for 24 hours. Then, they

629 were analyzed using the X-Gluc histochemical assay (Fermentas®), as described
630 by Jefferson et al. (1987), with minor modifications. After Gus staining, the
631 samples were clarified with graded ethanol series (30–70%), analyzed and
632 photographed.

633 **Quantitative PCR (RT-qPCR)**

634 Real-time PCR experiments were carried out using cDNA synthesized from total
635 RNA purified with TRIzol (Invitrogen®). Synthesis of first-strand cDNA was
636 performed by incubating 1 μg of total RNA with the M-MLV Reverse Transcriptase
637 (Promega) and a 24-polyTV primer (Invitrogen®). After synthesis, cDNAs were
638 diluted 10–100 times in sterile water for use in PCR reactions. Four biological
639 replicates from NT and GPX3s, under control, six or twelve hours after ABA
640 treatment, were performed for each experiment and three technical replicates
641 were performed for each reaction. All data analyses were performed after
642 comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method
643 (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). RT-qPCR reactions
644 were performed in an Applied Biosystems StepOne plus Real Time PCR system
645 (Applied Biosystems ®) using SYBR- genes are listed in **Table S9.**
646

647 **Germination Inhibition Assay**

648 For germination assays, NT and GPX3s (L.6, L.7 and L.8) seeds were surface-
649 sterilized in 4% (v/v) hypochlorite and then rinsed five times with sterile water.
650 Then, they were incubated on sterile MS basal medium (Murasnige and Skoog,
651 1962) containing 0.3% (w/v) phytagel and 0 or 5 μM ABA.

652 **Stomata aperture pattern**

653 Stomata closure quantitation was performed in 40-days-old plants shoots tissue.
654 Stomata were visualized by scanning electron microscopy and subsequently
655 quantified according to their aperture: completely open, partially open and
656 completely closed. Leaves of 40-day-old plants were sprayed with H_2O or 100
657 μM ABA and 24 hours post-treatment were detached and the samples prepared
658 as (Huang et al., 2009).

659 **Dark-induced Senescence experiment**

660 The senescence-induction experiments were performed as described by Kusaba
661 et al. (2007). Leave segments of approximately 5 cm from 40-day-old plants were
662 detached and maintained in the dark for seven days. The leaf fragments were
663 treated with the control solution (H_2O) or treated with 100 μM ABA or 100 μM 6-
664 benzylaminopurine (BAP) dissolved. Phenotype analyses were performed until
665 12 days after incubation and leave segments were collected after four days of
666 incubation for pigment quantitation and DAB staining experiments.

667 **ROS measurement**

668 Hydrogen peroxide was detected by 3,3'-diaminobenzidine (DAB) staining
669 (Thordal-Christensen et al., 1997). The leaves segments with approximately 5
670 centimeters, from 40-day-old plants, four days post-treatment with H_2O, 100 μM
671 ABA or 100 μM BAP, were detached and incubated overnight in DAB staining
672 solution (1 mg mL^{-1}) in the dark, at 28 °C with gentle agitation. Then, the leaves
673 were bleached with solution containing trichloroacetic acid, ethanol and
674 chloroform in the ratio 1,5 g :750 mL: 250 mL, respectively, until complete pigment
675 removal.

676 For histochemical detection of ROS, H_2DCFDA (2′,7′-Dichlorodihydrofluorescein
677 diacetate) staining was used. The leaves segments of 40-days-old treated, under
678 control or 24 hours after 100 μM ABA treatment, were incubated in 10 μM
679 H2DCFDA and were vacuum infiltered for 5 min. The leaves were then washed
680 with double distilled water and were observed under the confocal microscope
681 using laser beam of excitation 488 nm (Kristiansen et al., 2009). Measurements
682 of H_2O_2 content were performed by extracting H_2O_2 from leaves according to Rao
683 et al. (2000)using Ampliflu Red (Sigma-Aldrich) oxidation (Smith et al., 2004).
684 Fluorescence was monitored using a fluorometer at excitation and emission
685 wavelengths of 563 nm and 587 nm, respectively. Calibration was performed by
686 the addition of known quantities of H_2O_2.

687 **Pigment determination**

688 For total chlorophyll, chlorophyll a and b obtained after extraction in acetone were
689 determined spectrophotometrically at 663 and 649 nm. For total carotenoid, the

690 extract was monitored at 470 nm. The amount of pigment was calculated using
691 the equations proposed by (Lichtenthaler and Wellburn, 1983).

692

693 **Statistical Analysis**

694 The experiments were arranged in a completely randomized design in a 2x2
695 factorial: two genotypes (NT and GPX3s) and two treatments (control and ABA).
696 Six biological replicates were used to H_2O_2 content and four to RT-qPCR. Data
697 were plotted with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA,
698 USA) and analyzed using Student's test, and a p value ≤ 0.05 was considered to
699 be statistically significant. Each bar represents the average of replicates ± SEM.

700

701 **Conflicts of interest**

702 The authors have no conflicts of interest to declare.

703

704 **Contributions**

705 ALSP conducted all experiments, performed biochemical measurements,
706 interpretation and discussion of results, and paper writing. GP was co-advisor
707 and designed and obtained the transformed plants. DJM helped in biochemical
708 determinations. FCSN offered the infrastructure, and together with ALSP
709 conducted the proteomic analyses. MMP was the research supervisor and
710 assisted with writing and discussion of the manuscript.

711

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716 Tecnológico (FUNCAP) for financial support. J.A.G. Silveira and M. Margis-
717 Pinheiro are CNPq fellowship-honored researchers.

SUPPLEMENTAL FIGURES

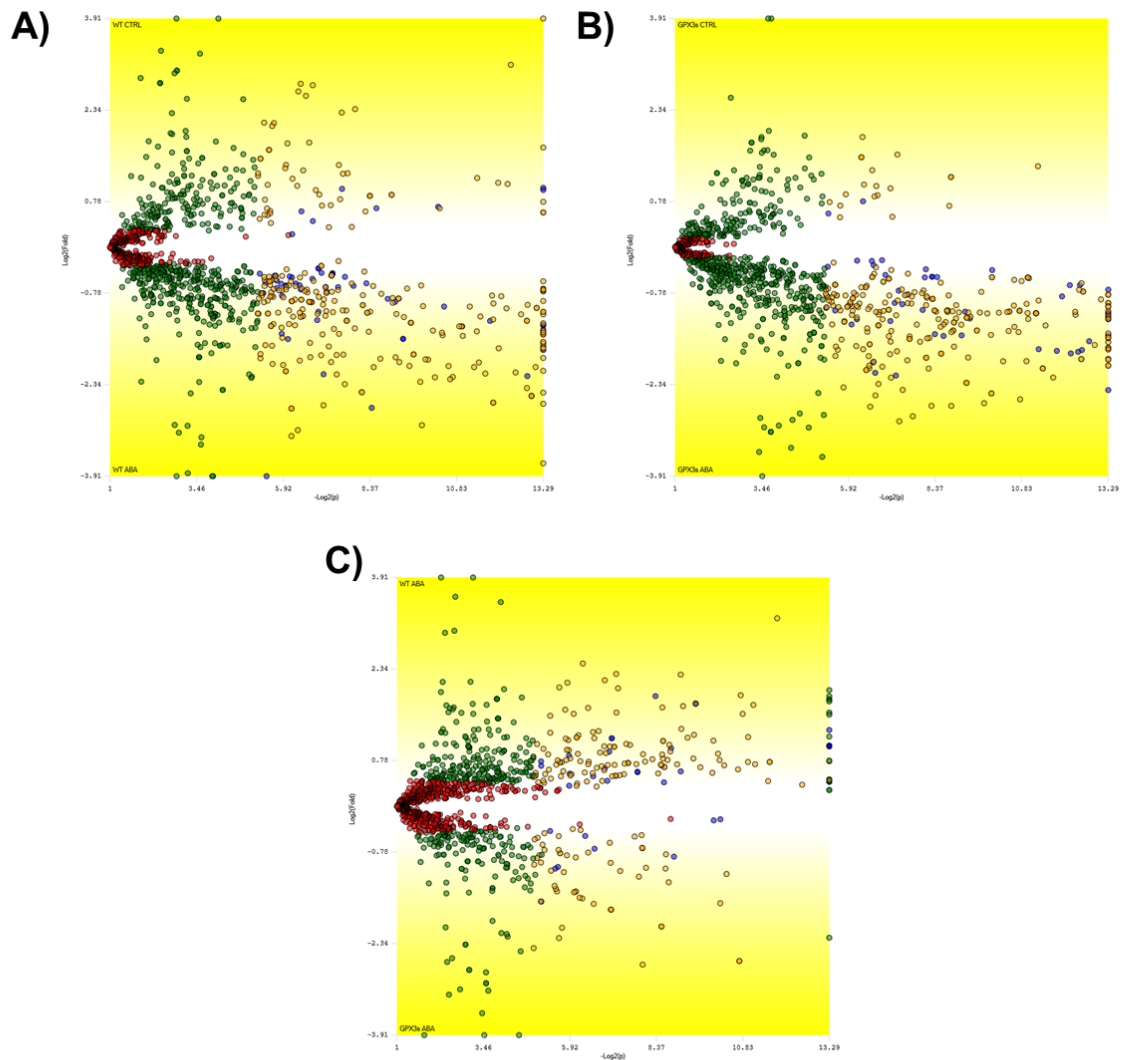
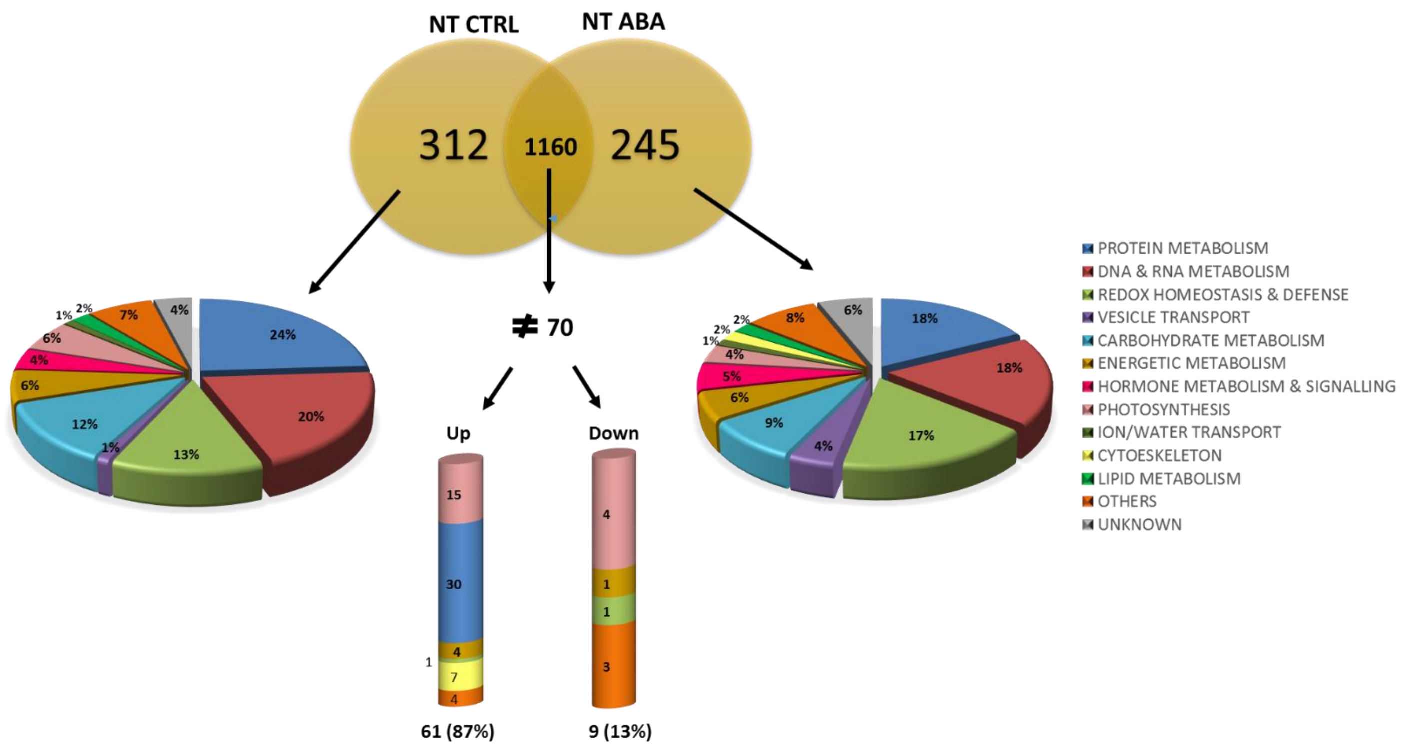
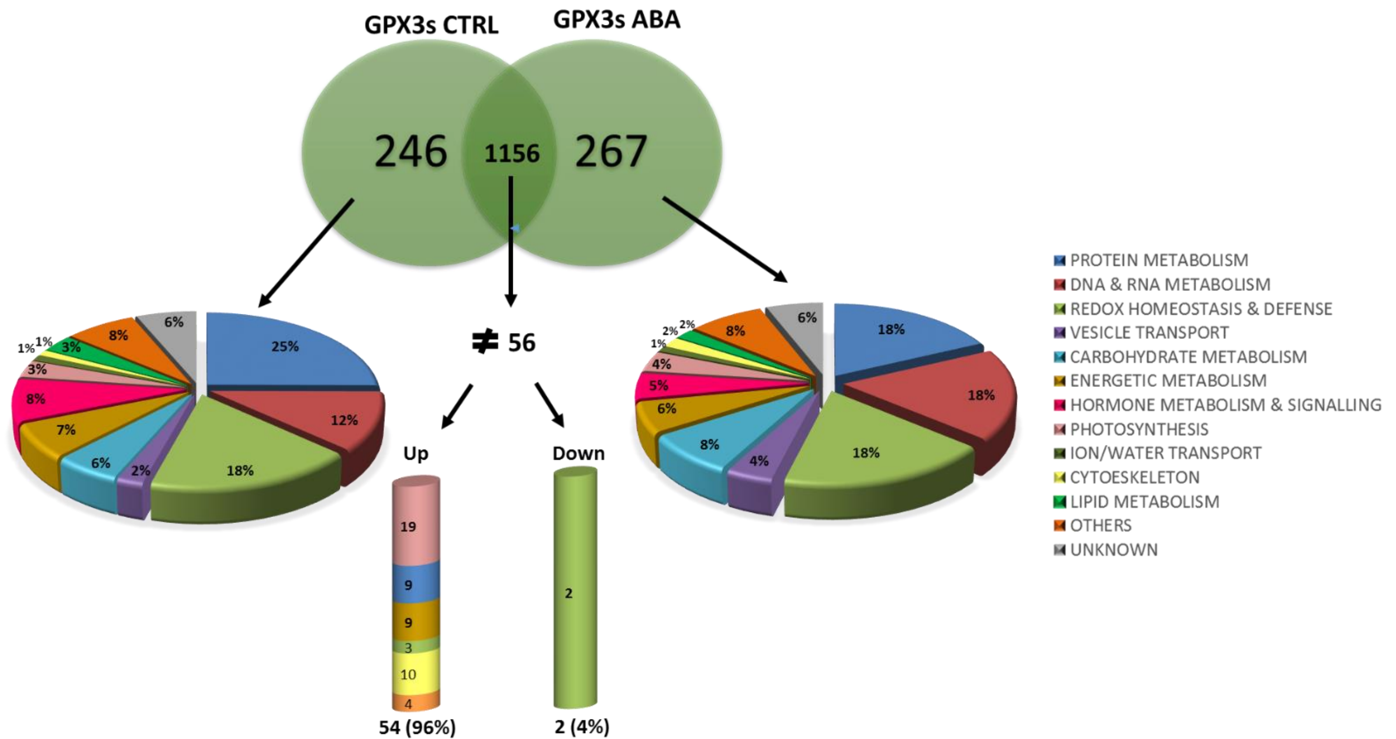


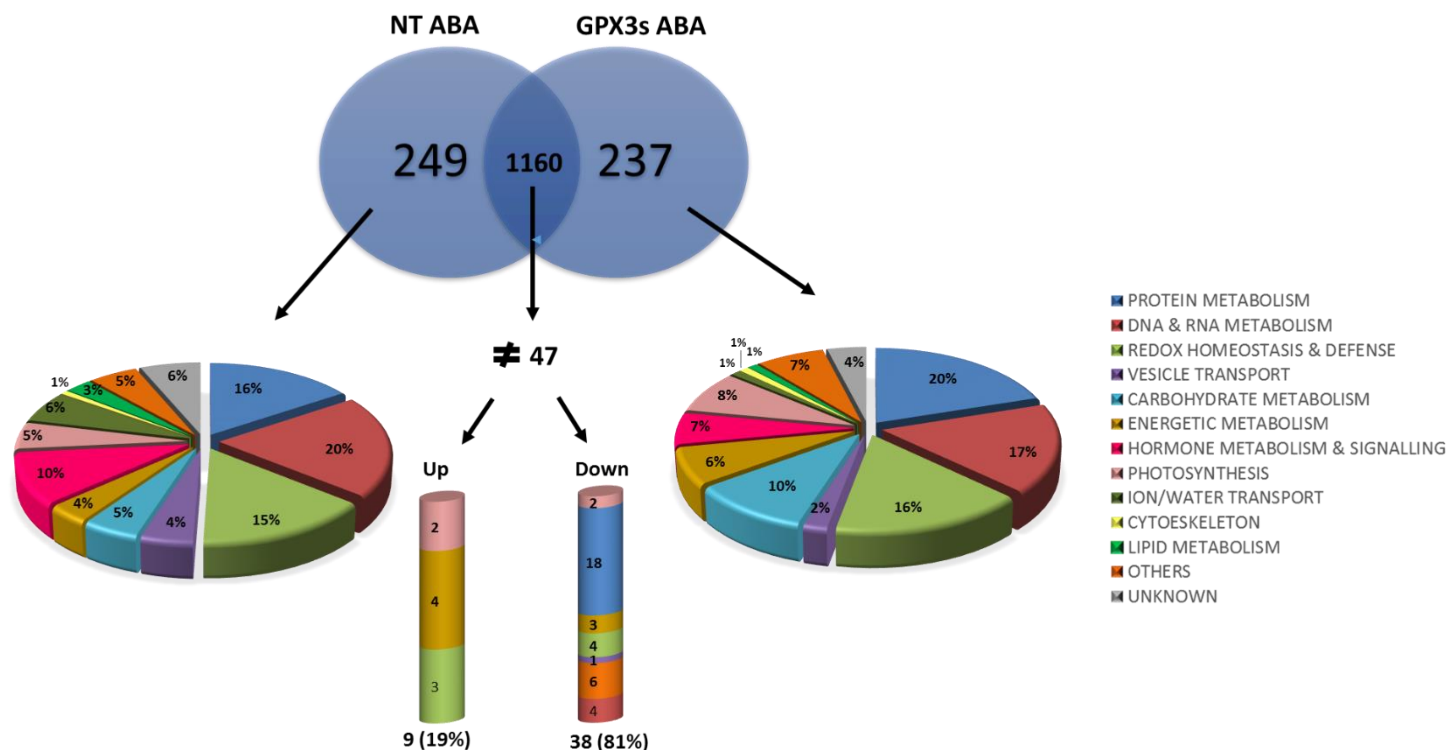
Figure S1. TFold pairwise analysis, where each protein is mapped as a dot on the plot according to its –$\log_2$(P-value) (x-axis) and $\log_2$(fold change) (y-axis). Red dots represent proteins that satisfy neither the fold change cutoff nor the FDR cutoff. Green dots are those that satisfy the fold-change cutoff but not FDR cutoff. Orange dots are those that satisfy both the fold change cutoff and FDR cutoff but are lowly abundant proteins and thus require further experimentation to certify their differential expression. Blue dots are proteins that satisfy all statistical filters**.** **A)** Comparison between NT under control conditions and NT 24 hours post-ABA treatment (442 red,353 green, 294 orange, 70 and blue dots). B) Comparison between GPX3s under control conditions and NT 24 hours post-ABA treatment (177 red, 633 green, 259 orange and 56 blue dots). **C)** Comparison between NT and GPX3s 24 hours post-ABA treatment (643 red, 417 green,182 orange and 47 blue dots).



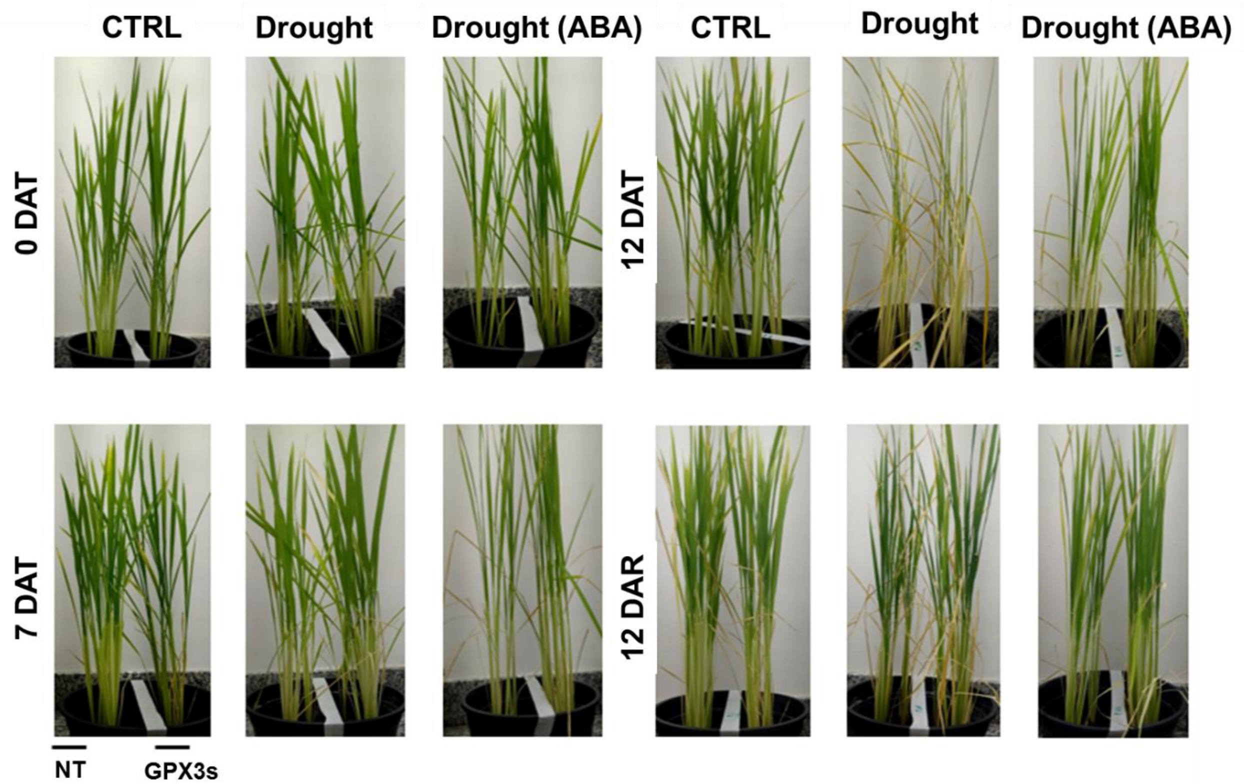
732 **Figure S2.** Venn diagram demonstrating the detected proteins overlap between
733 the non-transformed (NT) under control and 24 hours-post ABA treatment. There
734 were 312 and 245 unique proteins to NT control and ABA-treated, respectively.
735 Among the shared proteins (n=1160), 70 were differentially identified, 61 up and
736 9 down after ABA treatment, compared with control. Different colors represent
737 different biological processes, described in the legend in the right.



738 **Figure S3.** Venn diagram demonstrating the detected proteins overlap between
739 the GPX3s under control and 24 hours-post ABA treatment. There were 246 and
740 267 unique proteins to GPX3s control and ABA-treated, respectively. Among the
741 shared proteins (n=1156), 56 were differentially identified, 54 up and 2 down
742 GPX3s plants treated with ABA, compared with control. Different colors represent
743 different biological processes, described in the legend in the right.



744 **Figure S4.** Venn diagram demonstrating the detected proteins overlap between
745 the non-transformed (NT) and GPX3s 24 hours-post ABA treatment. There were
746 249 and 237 unique proteins to NT control and ABA-treated, respectively. Among
747 the shared proteins (n=1160), 47 were differentially identified, 9 up and 38 down
748 in GPX3s plants, compared with NT. Different colors represent different biological
749 processes, described in the legend in the right.



750 **Figure S5.** Phenotypic characterization of non-transformed (NT, left side in each
751 pot) and GPX3s (right side) plants submitted to drought stress. Plants under
752 control conditions and drought with ABA-treatment or not were observed 0, 7 and
753 12 days after treatment (DAT) and 12 days after recovery (DAR).
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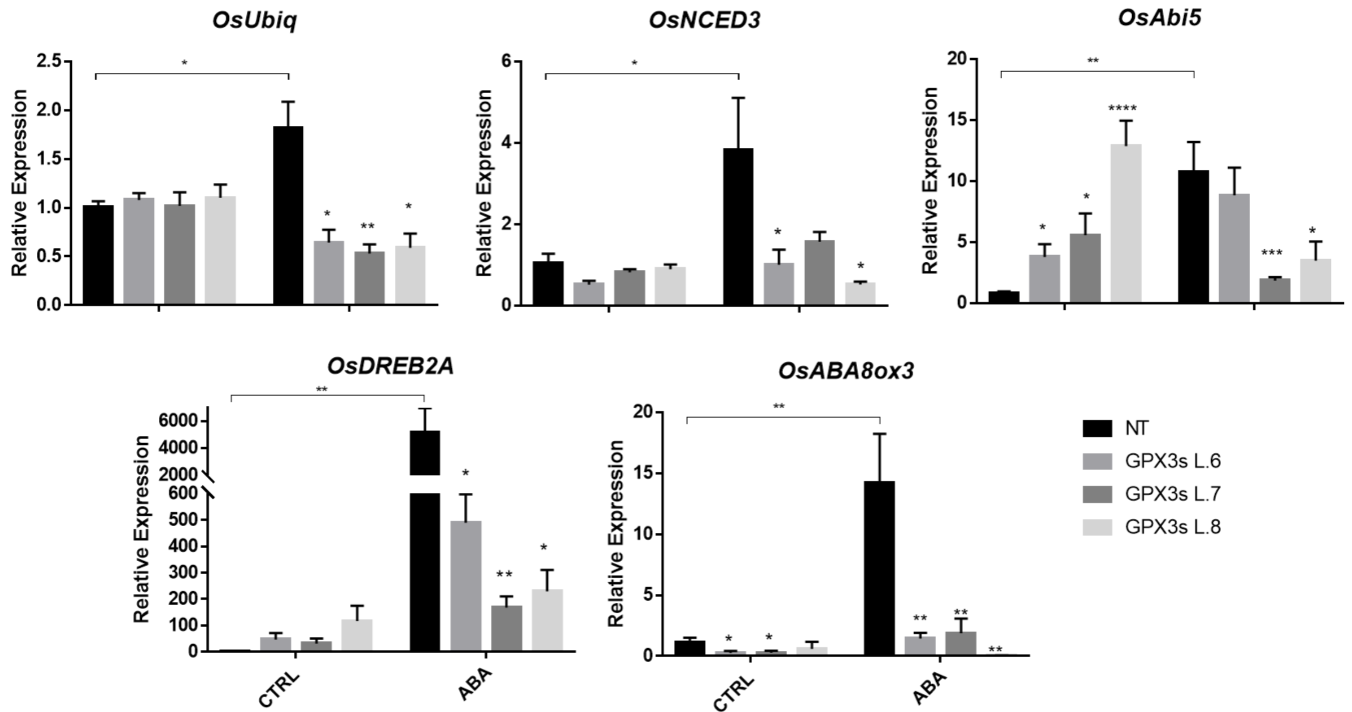

769 **Figure S6.** Transcript level of all OsGPX genes in non-transformed (NT) and GPX3s
770 (L.6, L.7 and L.8) plants cultivated in control or 6 hours after ABA treatment. (A) *OsUbiq*;
771 (B) *OsNCED*; (C) Os*Abi5*; (D) Os*DREB2A* and (E) Os*ABA8ox3*. The transcript level of
772 target genes in NT plants under control conditions was used to normalize transcript
773 accumulation in GPX3s and NT plants. Each bar represents the average of five replicates
774 ± SEM (n=4). Asterisks indicate significant differences at $p < 0.05$ compared to NT
775 in their respective conditions

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5. DISCUSSÃO GERAL

Os resultados mostrados no capítulo 1 revelaram a importância da isoforma mitocondrial de arroz (*OsGPX3*) na proteção contra ao estresse salino em plantas de arroz. De forma inesperada, as plantas silenciadas para o gene GPX3 não acumularam mais espécies reativas de oxigênio do que as plantas não-transformadas, entretanto tiveram menor taxa biomassa, conteúdo de clorofila e parâmetros de trocas gasosas após o estresse salino. Esses resultados sugerem que a GPX3 pode estar afetando na defesa contra a salinidade por outras vias de sinalização, atuando como gene chave na regulação de outros processos que são fundamentais para proteção do arroz.

Para entender as alterações induzidas pelo silenciamento do gene OsGPX3, a proteômica foi aplicada para investigar as proteínas diferencialmente acumuladas nas plantas GPX3s. O capítulo 2 nos revelou que a redução da expressão do gene GPX3 afetou diversos processos de sinalização, principalmente ligados à resposta ao ácido abiscísico, confirmando que esse gene atua em diferentes e que sua função vai muito além de simplesmente remover espécies reativas de oxigênio.

A proteômica não foi feita comparando os genótipos na condição controle e após tratamento com NaCl, entretanto, a partir da comparação entre NT e GPX3s na condição controle, foi possível detectar proteínas de grande importância para a defesa contra estresse salino com abundância reduzida em GPX3s, como: “Betaine aldehyde dehydrogenase”, “Hydroxyproline-rich glycoprotein-like”, “Drought-induced S-like ribonuclease”, several “Peptidylprolyl isomerases”, “Hypersensitive-induced response protein”, “Chaperones”, “Heat shock proteins” e , que foram exclusivamente encontradas nas plantas NT. Isso mostra que, apesar de não ter sido observado um maior acúmulo de ERO, as plantas GPX3s são deficientes de importantes proteínas que atuam na proteção contra o estresse osmótico.

Uma das principais estratégias que as plantas utilizam para responder aos efeitos do estresse osmótico induzido pelo estresse salino é o ajuste do potencial hídrico intracelular, diminuindo, assim, as diferenças entre os potenciais osmóticos entre o solo e as raízes. Esse mecanismo de ajuste osmótico possui

importante papel na prevenção da desidratação em plantas, da perda de turgor das células-guardas e da plasmólise celular (KOSOVÁ; PRÁŠIL; VÍTÁMVÁS, 2013), estando relacionado com o acúmulo de compostos osmoticamente ativos.

Para a célula, seria energeticamente menos dispendioso acumular íons de sais no citoplasma, entretanto, esse acúmulo promove muitos danos, como a degradação e inativação de muitas enzimas intracelulares. Assim, as plantas usualmente induzem a biossíntese de diversos compostos orgânicos hidrofílicos de baixa massa molecular como: compostos contendo nitrogênio (prolina, ácido glutâmico, glicina etc.), compostos quartenários de amônio (betaína e glicina betaína) ou poliaminas (espermina, espermidina, putrescina etc.); de diversos polialcóois de cadeia linear (D-ononitol, myo-inositol) ou de açúcares, como: glicose, frutose, trealose, rafinose, dentre outros (KOSOVÁ; PRÁŠIL; VÍTÁMVÁS, 2013; MUNNS, 2005). Outra possibilidade é o acúmulo de proteínas da superfamília LEA, como as dehidrinas, que não só diminuem o potencial osmótico, como também protegem outros componentes celulares dos efeitos adversos da desidratação (KOSOVÁ et al., 2011). Todos esses mecanismos de ajuste osmótico são bem conhecidos e descritos em plantas para lidar com efeitos negativos do estresse salino.

A prolina já é conhecida por possuir um papel crucial no ajuste osmótico e papéis alternativos têm sido sugeridos, como osmólito removedor de EROs e atuando como tampão redox ou chaperona molecular, estabilizando proteínas durante condições de estresse (ASHRAF; FOOLAD, 2007; VERBRUGGEN; HERMANS, 2008). De forma similar à prolina, a glicina betaína é um osmólito orgânico sintetizado por diversas famílias de plantas para balancear o potencial osmótico durante salinidade. Existem evidências de que esse osmólito também está envolvido com proteção de importantes enzimas e de estruturas da membrana (GUINN et al., 2011; RAZA et al., 2007).

O aumento na quantidade de proteínas envolvidas na síntese de prolina foram encontradas na halófita *Thellungiella*, não só sob condições salinas, mas, também, em condições controle (GONG et al., 2005; KANT et al., 2006; TAJI, 2004). O acúmulo de prolina livre foi reportado em *Pisum sativum* (NAJAFI et al., 2006), *Brassica juncea* (RAIS; MASOOD, 2013) e *Triticum aestivum*

(ASHFAQUE, 2014). Outros estudos mostraram que aplicação exógena de prolina e glicina betaína aumentou a tolerância da planta ao dano oxidativo induzido pelo sal por meio da intensificação do sistema de defesa e antioxidante (DEINLEIN et al., 2014; HASANUZZAMAN et al., 2014; IQBAL et al., 2014).

Além disso, os fitohormônios têm sido relatados como moléculas que podem influenciar na tolerância vegetal à salinidade por meio de modulação de diversos processos fisiológicos e mecanismos bioquímicos, que levam à adaptação das plantas à condições desfavoráveis e à mudanças na abundância de diversas proteínas (FATMA et al., 2013). Essas moléculas possuem importante papel na regulação dos processos de desenvolvimento das plantas sob estresses abióticos, erradicando ou reduzindo seus efeitos negativos (BARTOLI et al., 2013), e vêm sendo consideradas como uma novo meio de melhorar a qualidade da tolerância ao estresse em plantas, aliviando os danos do estresse e aumento a qualidade e a produtividade dos cultivares (IQBAL et al., 2014; QIU et al., 2014).

O ABA é um hormônio de estresse que regula muitos processos fisiológicos e tem papel crítico na regulação do *status* hídrico vegetal, controlando a perda de água das folhas, por meio da abertura e fechamento dos estômatos, e na indução de genes e proteínas envolvidos na tolerância à desidratação (ZHANG et al., 2006). Esses mecanismos de atuação do ABA estão intrinsecamente relacionados com a sinalização por Ca^{2+}, induzindo mudança na abundância de proteínas responsivas a ABA, que auxiliam na proteção da planta contra o estresse salino (KOSOVÁ; PRÁŠIL; VÍTÁMVÁS, 2013). O acúmulo de ABA tem sido observado em halófitas como *Thellungiella salsuginea* sob condições salinas (TAJI, 2004). GURMANI et al. (2013) reportou que o hormônio ABA está relacionado com a redução do conteúdo de Na^{+} e Cl^{-} e da razão Na^{+}/K^{+}, e com aumento no conteúdo de K^{+}, Ca^{2+}, prolina e açúcares solúveis em *Oryza sativa*. Em *Sorghum bicolor*, o ABA atrasou os efeitos deletérios do NaCl e promoveu maior tolerância ao estresse iônico (AMZALLAG; LERNER; POLJAKOFF-MAYBER, 1990; MAKELA et al., 2003).

Assim, a alteração de proteínas-chave para proteção ao estresse osmótico e a influência direta na via de sinalização do ABA podem explicar a

sensibilidade das plantas GPX3s ao estresse salino. Além disso, conforme discutido densamente nos artigos dos capítulos 1 e 2, esses resultados corroboram com a grande gama de possibilidades de atuação das enzimas glutationa peroxidases em diferentes vias, sejam elas redox ou de sinalização. Esses resultados vão ao encontro da recente discussão prosposta por FOYER et al. (2017) de investigar mais o estresse oxidativo pela visão da sinalização oxidativa e as complexidades que envolvem esses processos do que simplesmente analisar os danos causados pelo acúmulo de espécies reativas de oxigênio.

6.CONCLUSÕES

O gene *OsGPX3* tem um papel crucial na regulação de alguns processos epigenéticos e de resposta a hormônios, como o ácido abiscísico. Essa importante função pode explicar o fato das plantas GPX3s terem sido mais sensíveis ao estresse salino comparadas às plantas NT, haja vista que inúmeras classes de proteínas foram alteradas. O efeito do silenciamento de uma isoforma mitocondrial em vários parâmetros fotossíntéticos, sob estresse salino, confirmam a sinalização cruzada que ocorre entre cloroplastos e mitocôndrias. Esse é o primeiro estudo mostrando uma isoforma mitocondrial de uma enzima antioxidativa atuando em processos de sinalização tão complexos. Além disso, esse trabalho disponibilizou inúmeras proteínas candidatas que podem ser futuramente exploradas individualmente ou em conjunto para investigar com mais detalhes a atuação da GPX3 nesses processos.

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Anexo

Outros artigos publicados durante o doutorado

PNAS

A stress recovery signaling network for enhanced flooding tolerance in *Arabidopsis thaliana*

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Contributed by Julia Bailey-Serres, May 21, 2018 (sent for review March 5, 2018; reviewed by Mikio Nakazono and Su-May Yu)

Abiotic stresses in plants are often transient, and the recovery phase following stress removal is critical. Flooding, a major abiotic stress that negatively impacts plant biodiversity and agriculture, is a sequential stress where tolerance is strongly dependent on viability underwater and during the postflooding period. Here we show that in *Arabidopsis thaliana* accessions (Bay-0 and Lp2-6), different rates of submergence recovery correlate with submergence tolerance and fecundity. A genome-wide assessment of ribosome-associated transcripts in Bay-0 and Lp2-6 revealed a signaling network regulating recovery processes. Differential recovery between the accessions was related to the activity of three genes: *RESPIRATORY BURST OXIDASE HOMOLOG D, SENESCENCE-ASSOCIATED GENE113*, and *ORESARA1*, which function in a regulatory network involving a reactive oxygen species (ROS) burst upon desubmergence and the hormones abscisic acid and ethylene. This regulatory module controls ROS homeostasis, stomatal aperture, and chlorophyll degradation during submergence recovery. This work uncovers a signaling network that regulates recovery processes following flooding to hasten the return to prestress homeostasis.

flooding | ribosome footprinting | reactive oxygen species | dehydration | recovery

Plants continuously adjust their metabolism to modulate growth and development within a highly dynamic and often inhospitable environment. Climate change has exacerbated the severity and unpredictability of environmental conditions that are suboptimal for plant growth and survival, including extremes in the availability of water and temperature. Under these conditions, plant resilience to environmental extremes is determined by acclimation not only to the stress itself but also to recovery following stress removal. This is especially apparent in plants recovering from flooding. Flooding is an abiotic stress that has seen a recent global surge with dramatic consequences for crop yields and plant biodiversity (1–3). Most terrestrial plants, including nearly all major crops, are sensitive to partial to complete submergence of aboveground organs. Inundations that include aerial organs severely reduce gas diffusion rates, and the ensuing impedance to gas exchange compromises both photosynthesis and respiration. Additionally, muddy floodwaters can almost completely block light access, thus further hindering photosynthesis. Ultimately, plants suffer from a carbon and energy crisis and are severely developmentally delayed (4, 5). As floodwaters recede, plant tissues adjusted to the reduced light and oxygen in murky waters are suddenly reexposed to aerial conditions. The shift to an intensely illuminated and reoxygenated environment poses additional stresses for the plant, namely oxidative stress and, paradoxically, dehydration due to malfunctioning roots, frequently resulting in desiccation of the plant (6). Flooding can thus be viewed as a sequential stress where both the flooding and postflooding periods pose distinct stressors, and tolerance is determined by the ability to acclimate to both phases.

While plant flooding responses have been extensively studied, less is known about the processes governing the rate of recovery, particularly the stressors, signals, and downstream reactions generated during the postflood period. When water levels recede, it has been hypothesized that the combination of reillumination and reoxygenation triggers a burst of reactive oxygen species (ROS) production. Reoxygenation has been shown to induce oxidative stress in numerous monocot and dicot species (7–11) and related ROS production dependent on the abundance of ROS scavenging enzymes and antioxidant capacity of tissues (12–16). However, in the link between ROS and survival during recovery, several aspects remain vague, including the source of the ROS and whether it also has a signaling role. Mechanisms regulating shoot dehydration upon recovery also remain to be elucidated. In rice (*Oryza sativa*), the flooding tolerance-associated *SUB1A* gene also confers drought and oxidative stress tolerance during reoxygenation through increased ROS scavenging and enhanced abscisic acid (ABA) responsiveness (9). In *Arabidopsis*, ABA, ethylene, and jasmonic acid have been implicated in various aspects of postanoxic recovery (8, 16, 17). While these studies have furthered understanding of flooding recovery, the key recovery

PLANT BIOLOGY

Significance

Flooding due to extreme weather events can be highly detrimental to plant development and yield. Speedy recovery following stress removal is an important determinant of tolerance, yet mechanisms regulating this remain largely uncharacterized. We identified a regulatory network in *Arabidopsis thaliana* that controls water loss and senescence to influence recovery from prolonged submergence. Targeted control of the molecular mechanisms facilitating stress recovery identified here could potentially improve performance of crops in flood-prone areas.

Author contributions: E.Y., B.S., M.S., J.B.-S., L.A.C.J.V., and R.S. designed research; E.Y., D.V., A.L.S.P., M.H., T.R., A.S.-H., M.d.V., and J.B. performed research; E.Y., H.v.V., D.V., A.L.S.P., M.H., A.S.-H., M.d.V., R.C.S., J.B., and R.S. analyzed data; and E.Y., J.B.-S., L.A.C.J.V., and R.S. wrote the paper.

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Data deposition: The data reported in this paper have been deposited in the Sequence Read Archive (SRA) database, https://www.ncbi.nlm.nih.gov/sra (SRA accession no. SRP133870).

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Review Article

Interactions between plant hormones and heavy metals responses

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Abstract

Heavy metals are natural non-biodegradable constituents of the Earth's crust that accumulate and persist indefinitely in the ecosystem as a result of human activities. Since the industrial revolution, the concentration of cadmium, arsenic, lead, mercury and zinc, amongst others, have increasingly contaminated soil and water resources, leading to significant yield losses in plants. These issues have become an important concern of scientific interest. Understanding the molecular and physiological responses of plants to heavy metal stress is critical in order to maximize their productivity. Recent research has extended our view of how plant hormones can regulate and integrate growth responses to various environmental cues in order to sustain life. In the present review we discuss current knowledge about the role of the plant growth hormones abscisic acid, auxin, brassinosteroid and ethylene in signaling pathways, defense mechanisms and alleviation of heavy metal toxicity.

Keywords: ABA, auxin, brassinosteroid, ethylene, abiotic stress.

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