UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
DEPARTAMENTO DE GENÉTICA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ANÁLISE FUNCIONAL DOS GENES DE GLUTATIONA PEROXIDASE EM ARROZ (*Oryza sativa*) E ARABIDOPSIS (*Arabidopsis thaliana*)

GISELE PASSAIA

Porto Alegre
-Março de 2013-

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
DEPARTAMENTO DE GENÉTICA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ANÁLISE FUNCIONAL DOS GENES DE GLUTATIONA PEROXIDASE EM ARROZ (*Oryza sativa*) E ARABIDOPSIS (*Arabidopsis thaliana*)

GISELE PASSAIA

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular do Departamento de Genética da Universidade Federal do Rio Grande do Sul, como parte dos requisitos para a obtenção do título de Doutor em Ciências, área de concentração: Genética e Biologia Molecular.

Porto Alegre
-Março de 2013-

Orientadora

Prof. Dra. Márcia Pinheiro Margis

O presente trabalho foi desenvolvido no laboratório de Genética Vegetal do Departamento de Genética da Universidade Federal do Rio Grande do Sul (Porto Alegre), em colaboração com o Prof. Dr. Joaquim Albenísio Silveira do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará (Fortaleza), Prof. Dr. Jorge Mariath do Departamento de Botânica da Universidade Federal do Rio Grande do Sul (Porto Alegre) e a Professora Christine Helen Foyer do Centro de Ciência de Plantas, Universidade de Leeds, Reino Unido.

Fontes financiadoras

- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES);
- Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq);
- Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS);
- International Centre for Genetic Engineering and Biotechnology (ICGEB)

Agradecimentos

A minha orientadora, Profa. Dra. Márcia Margis, pelo suporte e incentivo na realização deste trabalho, por sempre acreditar no meu potencial e impulsionar para novas experiências profissionais. Obrigada por sempre ter uma palavra de ânimo e otimismo nos momentos de dúvida.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela concessão da bolsa.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa de doutorado sanduíche.

Ao PPGBM pela oportunidade de realizar o doutorado.

Agradeço com carinho, a todas as pessoas que de alguma forma contribuíram para execução deste trabalho, em especial:

Ao Elmo pela eficiência no seu trabalho, facilitando imensamente o nosso.

Ao professor Felipe dos Santos Maraschin por ser o relator e banca dessa tese pela cuidadosa e excelente revisão do manuscrito.

Aos membros da banca.

A todos do Laboratório de Metabolismo do Estresse de Plantas da Universidade Federal do Ceará representado pelo professor Joaquim Albenísio Silveira pela propagação de sementes de arroz e colaboração nos experimentos.

Ao Dr. Jorge Mariath, pela colaboração nos experimentos de análises de promotor.

A todos os amigos do Laboratório de Genética Vegetal pelo companheirismo, amizade, viagens, festas, risadas e discussão acalorada de dados experimentais.

À Dra. Christine Helen Foyer, por ter me acolhido em seu laboratório no período do meu doutorado sanduíche na Universidade de Leeds, Reino Unido. Aos colegas de laboratório e amigos que fiz durante esse período: Bélen Marquéz García, Gloria Comadira, Mercedez Hernándes Goméz, Guillaume Queval, Barbara Karpinska, Marian Quain e Daniel Schnaubelt.

A todos os amigos do Laboratório de Genomas e Populações de Plantas, pela amizade e ajuda durante esse período.

À Leila Fonini, aluna de iniciação científica, pela ajuda na execução de experimentos.

Aos amigos Rafael Arenhart, Lauro Bücker Neto e Pedro Beschoren da Costa, pelas longas conversas, risadas e ajuda durante o trabalho.

Às queridas amigas que sempre estarão no meu coração: Andréia Caverzan, Carolina Werner Ribeiro, Ana Paula Christoff e Fernanda Lazzarotto por toda a ajuda no meu crescimento profissional e pessoal, lindas viagens, conversas sem fim e amizade mais do que especial.

Ainda à Andréia pela maravilhosa parceria no trabalho e na amizade, por todos os conselhos e encorajamento nas decisões mais difíceis e complicadas. Obrigada por sempre estar presente, perto ou do outro lado do Atlântico.

Ao Marcelo Prietsch, meu amor, pelo apoio nas fases boas, mas especialmente nas ruins, raciocinando quando o pânico da mudança pra longe não me deixava fazê-lo. Por todo carinho e compreensão, sempre tendo uma palavra de otimismo e muito bom humor.

Aos amigos Camila Balbela Consoni e Marcelo Zepka Baumgarten, pela parceria inacreditável, risadas, viagens, carteados, sessões de cinema caseiro, praia, campo, acampamentos, atoladas em lugares inusitados e tantas outras coisa que animaram esses quatro anos de trabalho.

À minha amiga de infância, Jaqueline Panazzolo por sempre, sempre estar presente.

Aos meus pais Ivir e Marília, por sempre proporcionarem o que fosse necessário para a conclusão dessa etapa, pelo amor, carinho, compreensão e preocupação confortadoras. Muito obrigada. Amo vocês.

A Deus, pela sua presença real na minha vida.

“A fé e a razão (*fides et ratio*) constituem como que as duas asas pelas quais o espírito humano se eleva em contemplação da verdade”

João Paulo II

Sumário

Resumo

As espécies reativas de oxigênio (ERO) afetam significativamente a homeostase redox celular. No entanto, o peróxido de hidrogênio (H_2O_2), uma das ERO mais estudadas, é considerado regulador chave em uma série de processos fisiológicos, dependendo de sua concentração na célula: em baixas concentrações atua como molécula sinalizadora envolvida na aclimatação da planta a estresses, desencadeando tolerância a estresses bióticos e abióticos; por outro lado, em altas concentrações o H_2O_2 pode levar à morte celular. Gradientes de ERO e fitohormônios têm influência nas respostas de crescimento local e consecutivamente afetam o estado redox celular. Diversos fitohormônios afetam a sinalização redox celular controlando processos de crescimento e defesa. Os níveis de H_2O_2 intracelulares são controlados pela ação de diversas enzimas da classe das peroxidases e catalases. Dentre as peroxidases, a família de proteínas GPX pode ser encontrada em praticamente todos os reinos e vem sendo cada vez mais estudada em plantas. Em arroz, essa família é composta de cinco genes, enquanto em Arabidopsis foram identificados oito genes. Este trabalho teve como objetivo caracterizar funcionalmente as GPX mitocondriais e cloroplastídica de arroz e sete dos oito genes de *Arabidopsis thaliana.* Mutantes knockout de Arabidopsis para os genes *AtGPX1, AtGPX2, AtGPX3, AtGPX4, AtGPX6, AtGPX7* e *AtGPX8* foram obtidos. Nesta espécie, pelo menos *AtGPX2, AtGPX3* e *AtGPX6* são necessárias para a correta formação da arquitetura da raiz dependente dos hormônios ABA, auxina e SL. O sileciamento por RNAi em arroz para os genes *OsGPX1, OsGPX3* e *OsGPX4* gerou plantas com crescimento deficiente de raiz, parte área e formação de panículas. A redução em 80% da expressão gênica da isoforma cloroplastídica (*OsGPX4*) foi letal para o desenvolvimento de plântulas regeneradas a partir de calos de arroz, enquanto que a redução da expressão de 40% e 95% de *OsGPX1* (*GPX1s*) e *OsGPX3* (*GPX3s*), respectivamente, não impediu a regeneração de plantas. No entanto, plantas *GPX1s* apresentaram menor tamanho, menor número de panículas e menor produção de sementes em comparação com as plantas NT (não transformadas). Adicionalmente, plantas *GPX3s* apresentaram redução do comprimento tanto da parte aérea quanto das raízes, além de acúmulo de H_2O_2 cerca de vinte vezes maior do que a planta NT. Neste trabalho, foi demonstrada a participação das enzimas GPX durante o desenvolvimento vegetativo e reprodutivo de plantas de arroz e o estabelecimento da arquitetura da raiz dependente de hormônios em Arabidopsis. O conjunto de dados obtidos contribuem

para o entendimento do papel dessas enzimas na homeostase redox, como também na interação com fitohormônios nos processos de desenvolvimento e defesa vegetais.

Abstract

Reactive oxygen species (ROS) affect significantly cellular redox homeostasis. Hydrogen peroxide (H_2O_2), one of the most studied ROS, is considered a key regulator in a number of physiological processes dependent of its concentration in the cell: at low concentrations acts as a signaling molecule involved in plant acclimation to stress, triggering tolerance to biotic and abiotic stresses; on the other hand, at high concentrations can promote cell death. Gradients of ROS and phytohormones can influence the responses of local growth and consecutively can affect the cellular redox state. Several phytohormones affect redox signaling processes controlling cell growth and defense. The intracellular levels of H_2O_2 are controlled by the action of several enzymes like peroxidases and catalases. Among the peroxidases, the GPX family of proteins can be found in virtually all kingdoms and is being increasingly studied in plants. In rice, this family consists of five genes, while in Arabidopsis eight genes were identified. This study aimed to characterize functionally rice and Arabidopsis GPX genes. *Arabidopsis thaliana* nockout mutants for *AtGPX1, AtGPX2, AtGPX3, AtGPX4, AtGPX6,* and *AtGPX7 AtGPX8* genes were obtained. In Arabidopsis, at least *At*GPX2, *At*GPX3 and *At*GPX6 are necessary to hormone-dependent control of root achitecture formation. RNAi knockdown in rice of OsGPX1, and OsGPX3 OsGPX4 generated plants with shorter root and shoot lengths, and deficient panicle formation. An 80% gene expression reduction of the chloroplastic isoform (Os*GPX4*) was lethal, and impaired the regeneration of plants from rice *calli*, whereas the reduction of expression in 40% and 95% of Os*GPX1* (*GPX1s*) and Os*GPX3* (*GPX3s*), respectively, did not prevent plant regeneration. However, *GPX1s* plants were smaller and had fewer panicles and seed compared to NT (non-transformed) plants. Additionally, GPX3s plants showed reduced length of the shoot as roots and accumulation of H_2O_2 about twenty times greater than the plant NT. GPX participation during vegetative and reproductive development of rice plants and the hormone-dependent root arquitecture formation in Arabidopsis was demonstrated in this work. The data set obtained contributed to the understanding of the role of these enzymes in the redox homeostasis, as well as how the interaction with phytohormones in development processes and plant defense occurs.

LISTA DE ABREVIATURAS

ABA- ácido abscísico
APX – ascorbato peroxidase
AsA – ácido ascórbico ou ascorbato
CAT – catalase
cDNA – DNA complementar
DAB – tetrahidrocloreto de diaminobenzidina
DHA- dehidroascorbato
DHAR- dehidroascorbato redutase
eF – fator de alongamento
ERO – espécies reativas de oxigênio
GPX – glutationa peroxidase
GSH – glutationa
GSSH – glutationa oxidada
Gus – β-glucuronidase
MDAR- monodehidroascorbato redutase
MDHA – monodehidroascorbato
MS – meio de cultura de tecidos Murashige e Skoog
NO- óxido nítrico
NT – planta não-transformada
pb – pares de bases
PrxR – peroxirredoxina
RNA – ácido ribonucléico
RNAi – RNA de interferência
ROS – Reactive Oxygen Species
RT – transcriptase reversa
RT-qPCR – PCR quantitativo do produto da transcriptase reversa
SOD – superóxido dismutase
T-DNA – DNA transferido pela *Agrobacterium tumefaciens*
Ubi – ubiquitina
UV- radiação ultravioleta
X-Gluc – ácido 5-Bromo-4-chloro-3-indolyl b-D-glucuronico, substrato cromogênico do gene *Gus*

INTRODUÇÃO

Sinalização redox vegetal

A homeostase, ou balanço redox celular é um centro de integração de sinais, com papel fundamental na regulação do crescimento vegetal e defesa tanto em condições normais quanto em situações de estresse. O estado redox celular é a soma das moléculas ativas que promovem redução e oxidação, como também a capacidade antioxidante total do conjunto de moléculas antioxidantes. A razão entre as moléculas reduzidas em relação ao conjunto total de moléculas determina o estado redox celular. As espécies reativas de oxigênio (ERO) afetam significativamente a homeostase redox celular através da interação com diferentes componentes, tais quais ascorbato, glutationa (GSH), NAD(P)H e proteínas da superfamília das tiorredoxinas (Potters et al., 2010). A regulação redox é um mecanismo sensitivo usado pelas plantas com o objetivo de perceber e responder às perturbações do ambiente. Essas pertubações podem afetar as concentrações de ERO e consequentemente processos relacionados tanto ao crescimento vegetal quanto a respostas às mudanças nas condições ambientais (Potters et al., 2010). Além dos quatro antioxidantes mais conhecidos citados acima, outras moléculas antioxidantes, como os compostos fenólicos, quercetina, carotenóides, citocromos, tocoferóis e tocotrienóis, poliaminas, metabólitos secundários e proteínas contendo grupos-S redox-ativos, determinam o estado redox da célula interativamente (Potters et al., 2010; Foyer and Noctor, 2011).

O peróxido de hidrogênio (H_2O_2), o superóxido ($O_2^{\bullet-}$), o radical hidroxila (OH•) e o oxigênio singleto ($^1O_2$) são espécies reativas formadas continuamente a partir da redução univalente da molécula de oxigênio (O_2) como um subproduto do metabolismo aeróbico (para uma revisão ver Møller and Sweetlove, 2010). O H_2O_2 tem uma reatividade moderada, alta permeabilidade através das membranas e meia-vida longa de cerca de 1 ms, enquanto as demais EROs, $O_2^{\bullet-}$, OH• e $^1O_2$, possuem meia-vida muito mais curta (2-4µs; Gill and Tuteja, 2010). O H_2O_2 é considerado regulador chave em uma série de processos fisiológicos, dependendo de sua concentração na célula: em baixas concentrações atua como molécula sinalizadora envolvida na aclimatação da planta a estresses, desencadeando tolerância a estresses bióticos e abióticos; em altas concentrações pode levar à morte celular programada (Quan et al., 2008). O radical superóxido ($O_2^{\bullet-}$) é geralmente o primeiro tipo de ERO a ser gerado. Em tecidos

vegetais, aproximadamente 1-2% do consumo de O_2 é convertido em $O_2^{\bullet-}$(Puntarulo et al., 1988). Esse radical é produzido pela redução do O_2 durante o transporte de elétrons ao longo da rota não cíclica da cadeia transportadora de elétrons nos cloroplastos e outros compartimentos celulares vegetais. O maior sítio de produção do radical superóxido é o lado aceptor de elétrons do Fotossistema I (PSI) da membrana tilacóide (Gill and Tuteja, 2010). Por outro lado, o $^1O_2$ é o primeiro estado eletrônico excitado do O_2, é uma ERO atípica porque não está relacionado com a transferência de elétrons para o O_2, mas sim um elétron é elevado para um orbital de alta energia, dessa forma, liberando o oxigênio de seu estado restrito. A geração de $^1O_2$ durante a fotossíntese possui um forte efeito prejudicial nos fotossistemas (PS) I e II como também em toda a maquinaria fotossintética. Além disso, uma gama de estresses abióticos como salinidade e seca entre outros, levam ao fechamento dos estômatos e resultam em baixas concentrações intracelulares de CO_2 nos cloroplastos, o que favorece a formação do $^1O_2$ (Gill and Tuteja, 2010). Por fim, o radical OH• é considerado a ERO mais reativa conhecida, sendo responsável pela toxicidade do oxigênio *in vivo*. O radical OH• tem o potencial de reagir com todas as moléculas biológicas, tais como: DNA, proteínas, lipídios e quase qualquer constituinte celular. Devido à falta de um mecanismo enzimático para a eliminação deste radical altamente reativo, sua produção em excesso leva à morte celular em última instância (Vranová et al., 2002). Na presença de metais de transição como cobre e ferro, reações adicionais podem acontecer, por exemplo, através do mecanismo chamado Haber-Weiss ou pela Reação de Fenton liberando OH• e $O_2^{\bullet-}$, também pode reagir com outra espécie de radical livre, NO•, liberando peroxinitrito ($OONO^-$; Gill and Tuteja, 2010). A mitocôndria das células vegetais é um dos sítios produtores de ERO juntamente com os cloroplastos e peroxissomos (Rasmusson et al., 2004). A cadeia transportadora de elétrons da mitocôndria, ancorada na membrana interna, carrega energia suficiente capaz de reduzir diretamente o O_2, considerado fonte primária de geração de ERO mitocondrial (EROmt), acessório indispensável para a respiração aeróbica (Rhoads et al., 2006). A produção de EROmt normalmente é considerada inferior ao que é produzido pelos cloroplastos na presença de luz, durante a fotossíntese, ou peroxissomos no processo de fotorrespiração. No entanto, na ausência de luz ou em tecidos não verdes, mitocôndrias são a principal fonte produtora de ERO (Puntarulo et al., 1988). Aproximadamente 1-5% do consumo de O_2 mitocondrial leva a produção de H_2O_2 (Møller, 2001). Mitocôndrias de plantas modulam a produção de ERO gerada pela cadeia transportadora de elétrons através de

dois mecanismos que agem para manter o pool de ubiquinona em níves baixos. O primeiro, baseado na enzima oxidase alternativa (AOX), não é inibido pelo gradiente de prótons que atravessam a membrana interna e funciona quando a rota do citocromo é bloqueada (Finnegan et al., 2004). O segundo, baseado nas proteínas desacopladas (do inglês, *uncoupling proteins* – UCP), estas também são encontradas na membrana interna da mitocôndria e facilitam o vazamento de prótons através da membrana e, consequentemente, inibem a cadeia tranportadora de elétrons da mitocôndria (Hourton-Cabassa et al., 2004). A produção de ERO na mitocôndria ocorre durante condições normais de respiração, mas pode ser induzida por estresses bióticos e abióticos (Gill and Tuteja, 2010).

Os estresses abióticos ou bióticos levam a um aumento da produção de ERO, que em desbalanço podem reagir e danificar componentes celulares, mas as ERO são também importantes transdutores de sinal no controle da regulação de processos biológicos, tais como: crescimento, ciclo celular, morte celular programada, sinalização hormonal e desenvolvimento (Mittler et al., 2004). A produção de ERO, em muitos casos, é programada geneticamente, induzida durante o desenvolvimento e acionada por flutuações ambientais, afetando tanto o metabolismo primário quanto secundário. Efetivamente, o aumento do estado oxidado da célula é um componente essencial do repertório de sinais que as plantas utilizam para fazer ajustes apropriados de expressão gênica e estrutura celular em resposta ao ambiente e a fase de desenvolvimento (Foyer and Noctor, 2005b). Isso coloca as ERO além de apenas um subproduto tóxico do metabolismo celular, mas também como regulador chave nos processos de crescimento, desenvolvimento e defesa vegetais.

As ERO são geradas pela atividade de várias enzimas sendo as NAPH oxidases as mais estudadas. Organelas com atividade metabólica alta ou fluxo de elétrons intenso, como cloroplastos, mitocôndrias e peroxissomos, são as principais fontes de produção de ERO em plantas. As reações de detoxificação catalisadas pelos citocromos do citoplasma e retículo endoplasmático são fontes adicionais de produção de ERO (Urban et al., 1997). NADPH oxidases, peroxidases de parede celular pH-dependentes, oxalato oxidases do tipo germinina e amino oxidases são enzimas geradoras de ERO na membrana plasmática ou apoplasto (Hu et al., 2003; Walters, 2003).

Além dos sistemas não enzimáticos (Ascorbato, GSH, compostos fenólicos, entre outros) os organismos aeróbicos desenvolveram vários sistemas enzimáticos para equilibrar/neutralizar a geração de ERO. O sistema enzimático inclui um conjunto de

enzimas tais como superóxido dismutase (SOD), catalase (CAT), ascorbato peroxidase (APX), Peroxirredoxinas (PrxRs) e glutationa peroxidase (GPX; Shigeoka *et al.*, 2002). Esses sistemas enzimáticos antioxidantes podem ser encontrados em quase todos os compartimentos subcelulares, além disso, mais de uma enzima detoxificadora para uma ERO em particular pode ser encontrada em cada um dos diferentes compartimentos celulares, por exemplo: GPXs, PrxRs e APXs no citosol, cloroplastos e mitocôndrias, e CATs e APXs nos peroxissomos (Mittler et al., 2004). A figura 1 apresenta um esquema da participação das enzimas antioxidantes relacionadas com o metabolismo de EROs nos cloroplastos das células vegetais. A enzima SOD, presente em praticamente todos os compartimentos celulares, atua na linha de frente na defesa contra ERO, detoxificando o superóxido a peróxido de hidrogênio, podendo este ser eliminado pelas demais enzimas antioxidantes. Sendo assim, o balanço entre a concentração de SOD e a concentração das enzimas detoxificadoras de peróxido de hidrogênio é crucial para manter o equilíbrio entre os níveis de superóxido e peróxido de hidrogênio na célula (Mittler et al., 2004). A enzima catalase tem uma estrutura tetramérica com heme em seu sítio ativo, agindo na dismutação do H_2O_2 em H_2O e O_2, sendo indispensável para a detoxificação de ERO durante condições de estresse. Essa enzima possui uma das mais altas taxas de "turnover" entre todas as enzimas conhecidas: uma molécula de catalase é capaz de converter aproximadamente 6 milhões de moléculas de H_2O_2 em H_2O e O_2 por minuto. A catalase é importante na remoção do H_2O_2 gerado nos peroxissomos pelas oxidases envolvidas na β-oxidação dos ácidos graxos, fotorrespiração e catabolismo das purinas (Gill and Tuteja, 2010). APX pertece a uma classe de enzimas antioxidantes presentes exclusivamente em organismos fotossintetizantes (Viridiplantae, algae, euglena, por exemplo) e desempenha um papel fundamental na detoxificação das ERO. APX está envolvida na detoxificação do H_2O_2 provenientes dos ciclos Água-Água e Ascorbato-Glutationa, utilizando Ascorbato como doador de elétrons. As APX possuem alta afinidade pelo H_2O_2 (na ordem de μM), e provavelmente exercem um papel crucial no controle dos níveis das ERO durante o estresse (Gill and Tuteja, 2010). Peroxiredoxinas (PRXs) constituem o grupo mais recentemente identificado de enzimas detoxificadoras do peróxido de hidrogênio. As enzimas PRXs além de detoxificar H_2O_2 também decompõem hidroperóxidos do tipo alkyl e peroxinitritos, com diferenças de especificidade de substrato e propriedades cinéticas. Evidências experimentais sugerem uma tripla função para PRXs na biologia celular vegetal: a) antioxidante b) modulação

das vias de sinalização celular e c) sensor redox (Dietz et al., 2006). A participação de GPXs no metabolismo redox será descrita em detalhe posteriormente.

Os mecanismos de produção e detoxificação de ERO, seus alvos e suas funções em nível molecular precisam ser ainda melhor explorados. Estudos com mutantes nocaute para os diferentes genes codificantes das diversas enzimas detoxificadoras ou produtoras de ROS poderão esclarecer como os diferentes ramos da cadeia produtora/detoxificadora de ROS cooperam durante os estresses bióticos e abióticos. O conhecimento gerado poderá permitir desenvolver plantas de interesse agronômico com habilidade de detoxificar e/ou controlar os níveis de ERO excedentes na célula, e resistir às severas mudanças ambientais.

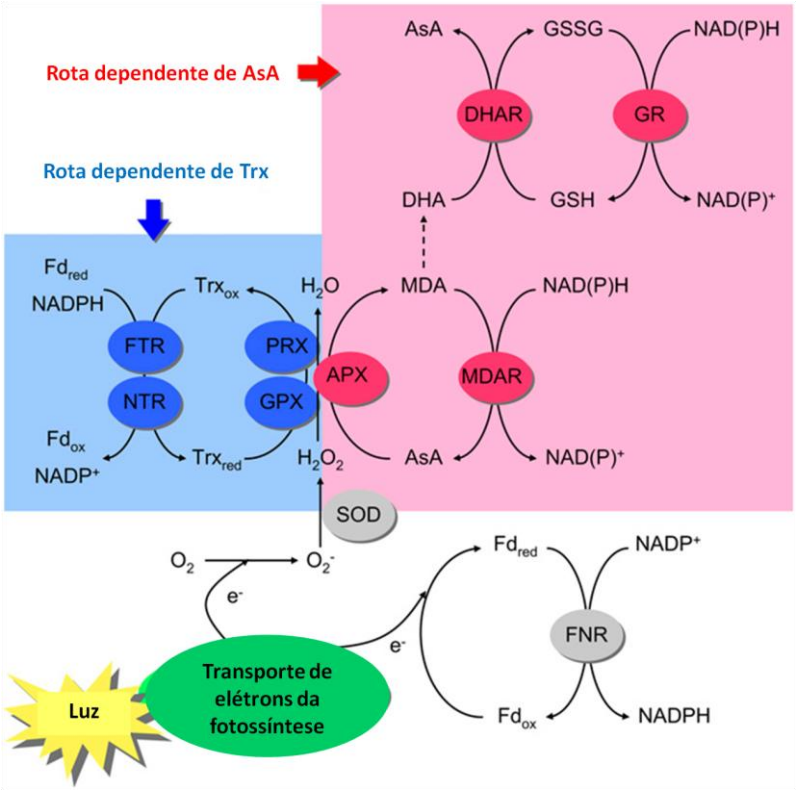


Figura 1. Ciclo do Ascorbato-Glutationa (à direita) e Tiorredoxina dependente (TRX/GPX, esquerda), rotas de detoxificação do H_2O_2 nos cloroplastos. AsA, ascorbato; Trx, tiorredoxina; Fd, Ferredoxina; FNR, Ferredoxina NADPH reductase; FTR, Ferredoxina tiorredoxina redutase, MDAR, Monodeidroascorbato redutase (Adaptado de Foyer & Shigeoka, 2011).

Interações entre hormônios e a sinalização redox

Gradientes de ERO e fitohormônios têm influência nas respostas de crescimento local agindo sobre o estado redox celular, entre outros aspectos. O conceito teórico de estado redox é definido como o grau de oxidação celular (Dietz, 2008). As ERO têm sido implicadas como mensageiros secundários em diversas respostas hormonais em plantas. Por exemplo, o H_2O_2 funciona como um componente à jusante da transdução de sinal mediada por auxina durante o processo de gravitropismo da raiz em milho (Joo et al., 2001). Muitos fitohormônios promovem produção de ERO, geralmente através da ativação de NADPH oxidases, ou ainda alteram hormônios que participam da sinalização redox induzindo tolerância a um grande espectro de estresses (Foyer and Noctor, 2009). Existem evidências de que diversos fitohormônios interagem com a sinalização redox celular controlando processos de crescimento e defesa em resposta aos estresses ambientais. Entre eles estão auxina, ácido salicílico, etileno, ácido jasmônico, strigolactonas, ácido abscísico e giberelinas (Bartoli et al., 2012).

Auxina

Auxinas, principalmente o ácidoindolacético (AIA), estão envolvidas no estabelecimento da polaridade do embrião, filotaxia, formação de órgãos, crescimento e desenvolvimento da raíz, promoção da formação dos pelos radiculares, formação de raízes laterais, espessura da folha, formação e distribuição das veias foliares (Casimiro et al., 2001; Leyser, 2005). As auxinas são também, como componentes das respostas morfogênicas, induzidas por estresse (SIMR – stress-induced morphogenic response; Potters et al., 2007; Potters et al., 2009). Além do aumento da produção de ERO afetar a homeostase de auxina, esta é também um gatilho para produção de ERO (Tognetti et al., 2012). Por exemplo, a indução do H_2O_2 por auxina atua como um sinal para a resposta gravitrópica da raiz e abertura dos estômatos (Joo et al., 2001; Song et al., 2006). Em milho as auxinas também induzem a produção do íon superóxido, facilitando o afrouxamento da parede celular durante o crescimento e alongamento celular (Schopfer et al., 2001). Além disso, o estado redox do *pool* de ascorbato no apoplasto afeta a sensibilidade e a resposta à auxina (Pignocchi et al., 2006). O mesmo pode ser afirmado para dois importantes reguladores da homeostase redox, NADPH-linked thioredoxin (NTR) e GSH, os quais alteram o transporte e o metabolismo de auxina. O mutante triplo *ntra ntrb cad2* de *Arabidopsis thaliana*, defectivo nos dois componentes

citados acima apresentam perda de dominância apical, diminuição do número de raízes laterais, defeitos vasculares entre outros fenótipos relacionados à auxina, ligados à diminuição do número de transportadores de auxina PIN1 no meristema radicular (Bashandy et al., 2010). Adicionalmente, a regulação redox de GSH está envolvida parcialmente no crescimento da raiz através do controle da expressão das proteínas PIN (*PIN-formed*) 1, PIN2 e PIN7 no ápice da raiz (Koprivova et al., 2010). O efeito do tratamento com auxina no estado redox do *pool* do ascorbato em raízes de tomateiro aumenta a forma oxidada do dehidroascorbato (DHA) e assim promovendo a retenção do crescimento da raiz, e também a produção de raízes laterais, uma resposta morfogênica ligada ao estresse oxidativo (Tyburski et al., 2007).

Strigolactonas

Strigolactonas (SLs) são moléculas sinalizadoras sintetizadas nos plastídeos a partir da rota dos carotenóides, principalmente nas partes mais baixas do caule e nas raízes (Domagalska and Leyser, 2011). Esses fitohormônios são considerados como mensageiros secundários das rotas de sinalização por auxina, interagindo umas com as outras (auxinas e SLs) em um *feedback* dinâmico no controle do desenvolvimento de órgãos. As SLs atuam restringindo o transporte de auxinas de maneira sistêmica e local, causando o acúmulo de auxina em níveis que inibem o crescimento, por exemplo, em brotos, controlando a ramificação de brotos axilares (Gomez-Roldan et al., 2008; Umehara et al., 2008; Ruyter-Spira et al., 2011). Desta forma, é provável que a rota de sinalização gerada por SLs produzam ERO como mensageiros secundários da mesma forma que auxinas e outros fitohormônios (Bartoli et al., 2012). Alguns estudos têm mostrado que SLs interagem diretamente com as rotas de sinalização redox (Woo et al., 2004), mas a natureza dessa interação ainda não é completamente entendida. Pode-se citar o seguinte exemplo: o mutante *ore9*, com atraso no processo de senescência, é mais tolerante ao estresse oxidativo que o tipo selvagem. ORE9 é um componente importante da rota de sinalização de SL, também relatado na literatura como MAX2 (Woo et al., 2001; Stirnberg et al., 2002).

Ácido Abscísico

O fitohormônio ácido abscísico (ABA) é produzido em plantas por um via indireta envolvendo a clivagem oxidativa da molécula *9-cis-epoxycarotenoid* (C_{40}) produzindo xantina (C_{15}) e o subproduto C_{25} proveniente da clivagem de carotenóides (Schwartz et al., 2003). ABA age como um regulador positivo da senescência foliar, acumulando em resposta a estresses que envolvem déficit de água, como seca, sal ou temperaturas extremas levando a uma reprogramação global da expressão gênica e de processos adaptativos como fechamento de estômatos e acúmulo de solutos osmo-compatíveis (Chandrasekar, 2008). Além disso, ABA ativa NADPH oxidases, que levam à produção de ERO, importantes na mediação do fechamento de estômatos e na regulação das cascatas mediadas por MAP Kinases (Guan et al., 2000; Pei et al., 2000; Zhang et al., 2001). Outra evidência que suporta a ideia de que existe uma associação entre sinalização redox e rotas de sinalização por ABA provém dos estudos com os mutantes *vtc1* e *vtc2* de Arabidopsis que acumulam níveis baixos de ascorbato comparados à planta selvagem (Kiddle et al., 2003; Pastori et al., 2003; Kerchev et al., 2011). Esses mutantes possuem níveis mais elevados de ABA comparados com a planta selvagem, além de haver uma reprogramação da expressão gênica que é característica de respostas de sinalização de ABA (Pastori et al., 2003). É interessante ressaltar também que o acúmulo de ABA modula o transporte de auxina na ponta da raiz aumentando secreção de prótons para a manutenção do crescimento da raiz sob estresse moderado de seca (Xu et al., 2012).

Evidências cumulativas suportam o conceito de que sinalização hormonal e a sinalização celular redox formam uma rede integrada que regula rotas relacionadas ao crescimento vegetal e respostas de defesa (Bartoli et al., 2012).

Glutationa peroxidase

GPXs pertencem a uma família, presente em todos os reinos, que inclui tiol-peroxidases não-heme, as quais catalisam a redução de H_2O_2 ou hidroperóxidos orgânicos a água ou a alcoóis correspondentes. Em mamíferos foram descritos sete genes classificados como GPX baseado na similaridade das seqüências, nas funções bioquímicas de seus produtos gênicos, e na presença de uma tríade catalítica formada por selenocisteína/cisteína, glutamina e triptofano. A maioria das enzimas GPX de mamíferos são selenoproteínas, pois essas usam selenocisteína (Sec) no sítio catalítico e glutationa como substrato redutor (GSH). O aminoácido selenocisteína (Sec), é

codificado por um códon de parada UGA que é reconhecido por um Sec- t-RNA específico (Herbette et al., 2007; Toppo et al., 2008). O uso de selênio e glutationa, característico de GPXs canônicas, na verdade, parece ser uma aquisição recente no curso da evolução, especialmente encontrada em vertebrados e mamíferos, os quais possuem ainda estruturas oligoméricas. A figura 2 apresenta resumidamente o mecanismo de redução peroxidativa de GPX do tipo NS (non-selenium).

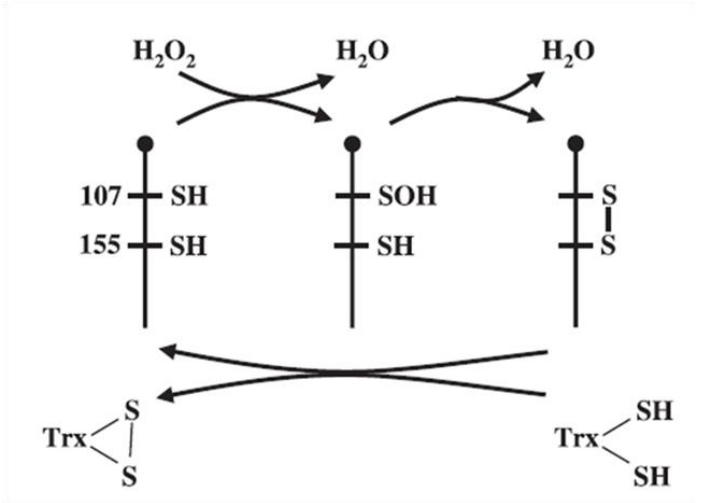


Figura 2. Mecanismo de redução peroxidativa de GPX do tipo NS (Non-Selenium). O mecanismo de redução do H_2O_2 e a regeneração de GPX utilizando tiorredoxina são propostos para *Brassica napus*. As proteínas são representadas por traços e a extremidade N-terminal representada por um círculo fechado (Retirado de Herbette et. al, 2007.

Embora inicialmente tenha se pensado que a maioria das GPXs utilizassem glutationa como redutor, evidências recentes sugerem que um grande número de GPX tenha especificidade por tiorredoxina (Toppo et al., 2009). Foi proposto (Toppo et al., 2008) que essas proteínas sejam renomeadas para "thioredoxin GPX-like peroxidases' (TGPX), já que a nomenclatura dessa superfamília é baseada no nome do substrato doador de elétrons ao invés do ancestral comum (GPX de classe 4 de mamíferos). Os mesmos autores afirmam que os requisitos mínimos que diferem peroxidases baseadas em tiorredoxina de GPXs verdadeiras são as seguintes: a) estrutura monomérica ou dimérica, b) um sítio ativo, peroxidativo, com cisteína (CxxC), c) uma cisteína exposta e resolvida no bloco delimitando a hélice funcional da proteína ativa, a qual após a oxidação se arranja em uma ponte dissulfídica intramolecular com a cisteína

peroxidativa. Sequências de TGPX são encontradas em Artrópodes, Euglenozoa, Fungos, Viridiplantae e Bactéria (Toppo et al., 2008).

O papel das GPX não se limita a defender os organismos contra o estresse oxidativo, pois essas enzimas participam também de complexas cascatas de sinalização. Por exemplo, a formação de estruturas tetraméricas assumidas por alguns dos membros dessa superfamília pode modular finamente o estado redox da célula em resposta ao excesso de H_2O_2 ou hidroxiperóxidos (ou ambos). A GPX3 de levedura, por exemplo, pode especificamente ativar o fator de transcrição Yap-1 (Delaunay et al., 2002). Portanto, novas funções têm sido atribuídas a essa superfamília e seu papel original no metabolismo celular deve ser revisto. As GPX estão envolvidas em diversos processos fisiológicos importantes, sendo a manutenção de sua atividade essencial para o perfeito funcionamento da célula (Méplan et al., 2008). A falta ou a redução da atividade das GPXs pode estar na base do desenvolvimento de doenças e também do envelhecimento (Méplan et al., 2008). Nesse contexto foi verificado que camundongos com nocaute do gene de GPX de classe quatro morrem no útero, confirmando a importância dessas enzimas para o desenvolvimento nos mamíferos. O fenótipo letal pode ser recuperado pela superexpressão de um transgene de GPX4 que protege as células animais do dano oxidativo (Méplan et al., 2008). Existem evidências em humanos de que o aumento das ERO, o dano molecular resultante deste aumento assim como a ativação de vias anti-inflamatórias contribuem para o envelhecimento. Quando a atividade de GPX diminui dentro da célula, ocorre um aumento na concentração de H_2O_2, resultando na ativação de fatores nucleares da via anti-inflamatória. Alguns estudos em populações adultas sugerem um decréscimo idade-dependente da atividade das enzimas antioxidantes, consistente com a hipótese de que o aumento de radicais livres contribui para o envelhecimento (Espinoza et al., 2010).

Em um trabalho prévio nosso grupo realizou um estudo das relações evolutivas envolvendo mais de 200 seqüências de GPX derivadas de um grupo muito diverso de organismos (Margis et al., 2008). Essas análises revelaram que as classes 1, 2, 3, 5, e 6 das GPXs de vertebrados têm origem filogética comum e provavelmente distinta das GPX de classe 4. Em nossas análises, GPX4 agrupou com seqüências de plantas e fungos. No entanto, não foi possível determinar se uma seqüência precursora dessa classe de GPX já estaria presente antes da divergência entre vertebrados e invertebrados ou se a origem desses genes é polifilética. Neste último caso, as GPX4 estariam submetidas a fortes forças seletivas que provavelmente ditaram uma convergência

funcional de grupos taxonomicamente bastante distantes. Além das análises filogenéticas demonstrando a alta relação entre as GPX4 de vertebrados e as GPX de plantas, esses estudos revelaram que esses dois grupos de GPX possuem organização gênica bastante divergente, reforçando a hipótese de uma história evolutiva independente com convergência funcional.

Em arroz, foi identificada uma família multigênica de glutationa peroxidases formada por cinco genes através de análises *in silico*. Essas análises predizem hipoteticamente a localização das proteínas de GPx de arroz no cytosol (OsGPX2 e OsGPX5), mitocôndria (OsGPX1 e OsGPX3) e cloroplasto (OsGPX4; Margis et al., 2008). Em *Arabidopsis thaliana*, GPXs são codificadas por uma família gênica formada por oito membros. AtGPX foram preditas como localizadas no citosol (AtGPX2, AtGPX4, AtGPX6 e AtGPX8), cloroplasto (AtGPX1 e AtGPX7), mitocôndria (AtGPX3) e retículo endoplasmático (AtGPX5; Milla et al., 2003). Até o momento, o papel das isoformas de GPX de arroz ainda não foi avaliado, enquanto que GPX de *Arabidopsis* possuem alguns poucos trabalhos. Sabe-se, por exemplo, que a isoforma GPX3 de *Arabidopsis thaliana* interage fisicamente com uma proteína fosfatase do tipo 2C (PP2C) (ABI2) e de maneira menos eficiente com ABI1. ABI2 por sua vez ativa canais de Ca2+ e K+ da membrana plasmática permitindo o fechamento de estômatos (Miao et al., 2006a). Além disso, duas GPXs estromais (GPX1 and GPX7) parecem ser importantes nas funções do cloroplasto, particularmente no processo de aclimatação luminosa e nas respostas de defesa imune da planta (Chang et al., 2009). O mutante de *Arabidopsis gpx8* mostrou aumento no dano de DNA, sugerindo participação dessa proteína na proteção do núcleo (Gaber et al., 2012). Todos esses estudos mostram a participação de AtGPX em importantes funções, mas a função de cada membro dessa família ainda não é conhecido totalmente. Portanto, novas funções têm sido atribuídas a essa superfamília e seu papel original no metabolismo celular deve ser revisto e estudado em maior detalhe, especialmente nas plantas modelo de estudo para genética molecular vegetal, *Oryza sativa* e *Arabidopsis thaliana*.

Modelos vegetais para o estudo da genética molecular vegetal

Oryza sativa L.

O arroz é considerado a planta modelo, entre as monocotiledôneas, para estudos biológicos. Possui genoma relativamente pequeno (390 Mb) comparado ao genoma de outros cereais como o milho, cevada e trigo que têm seus genomas estimados em 3000, 5000 e 16000 Mb, respectivamente. Em 2005, o consórcio *International Rice Genome Projetc* (IRGSP, 2005) sequenciou e publicou uma versão do genoma do arroz. Dois anos depois, em 2007, uma anotação acurada do genoma de *Oryza sativa* L. *ssp*. *japonica* foi publicada, estimando em aproximadamente 32.000 genes a composição do genoma (The Rice Annotation Project, 2007). Ferramentas que reforçam o papel do arroz como planta modelo estão disponíveis, tais quais: mapa genético saturado (International Rice Genome Sequencing Project, (http://rgp.dna.affrc.go.jp/IRGSP/), metodologia eficiente de transformação genética (Hiei et al., 1994) e mapas genéticos comparativos entre os cereais (Moore et al., 1995). Foi demonstrada a existência de regiões extensas de genoma conservadas e devidamente ordenadas entre os cereais; ou seja, a existência de intensa sintenia entre as gramíneas (Moore et al., 1995). O trigo, o milho e o arroz juntos são os cereais mais cultivados no mundo, perfazendo cerca de 50% das calorias consumidas pela população (Vij et al., 2006).

Cerca de 150 milhões de hectares de arroz são cultivados anualmente no mundo, chegando a uma produção de cerca de 590 milhões de toneladas. Na maioria dos países em desenvolvimento, o arroz é a cultura alimentar de maior importância, constituindo-se como alimento para 2,4 bilhões de pessoas. A Ásia é a maior produtora de arroz, concentrando cerca de 80% da produção mundial (http://www.agencia.cnptia.embrapa.br). O Brasil ocupa a nona colocação na produção mundial de arroz com cerca de 11,2 milhões de toneladas. A cultura do arroz ocupa a quinta posição no Brasil entre as culturas mais plantadas (http://www.faostat.fao.org). O estado do Rio Grande do Sul é o maior produtor dentre os estados brasileiros, com aproximadamente 68% da produção nacional (Gomes & Magalhães Júnior, 2004). A produção do cereal é oriunda do sistema de cultivo de várzea e de terras altas.

É importante ressaltar, que o aumento da produtividade das culturas, especialmente a do arroz, está relacionado com a capacidade das cultivares de se adaptarem, como também de resistir às condições ambientais adversas. Desse modo, a

elucidação das funções de genes relacionados ao estresse oxidativo, torna-se estratégia importante para o desenvolvimento de plantas mais adaptadas ao ambiente.

Arabidopsis thaliana L.

Arabidopsis thaliana é uma erva pertencente à família Brassicaceae e é indiscutivelmente o modelo vegetal mais utilizado em estudo de genética molecular vegetal. Atualmente o gênero Arabidopsis possui nove espécies listadas e mais oito subespécies reconhecidas (O'Kane and Al-Shehbaz, 1997; Steve L. O'Kane, 2003). Lista-se uma série de características que a tornam um excelente modelo de estudo: a) curto ciclo de vida, em média de quatro a seis semanas, e possibilidade de produção de mais de 10.000 sementes por planta adulta; b) tamanho reduzido, possibilitando o cultivo de dezenas de plantas em um pequeno espaço; c) fácil adaptação, com crescimento em meio de cultura estéril, além do cultivo de células isoladas com a possibilidade de regeneração de novos indivíduos a partir dessas; d) autofecundação: permitindo que novas mutações apareçam em homozigosidade em pouco tempo; e) susceptibilidade de infecção por *Agrobacterium tumefaciens*; f) genoma de tamanho pequeno (167 milhões de pares de bases, 25.706 genes) (Raven, 2007). Nas últimas três décadas, Arabidopsis se tornou organismo modelo de pesquisa nas áreas de fisiologia, desenvolvimento, bioquímica e biologia molecular. Os genes de Arabidopsis têm sido amplamente estudados através da genética reversa. Um grande número de banco de mutantes gerados pela inserção de uma sequência de DNA exógeno em sequências aleatórias do genoma, visando interromper genes e avaliar o fenótipo da planta tem sido gerados (Azpiroz-Leehan and Feldmann, 1997; Krysan, et al., 1999; Speulman et al., 1999; Tissier et al., 1999; Sussman et al., 2000; Sessions et al., 2002; Alonso et al., 2003). Com essa extensa disponibilidade de material genético, Arabidopsis, constitui um modelo vegetal muito útil para estudo de genômica funcional.

OBJETIVOS

Este trabalho teve como objetivo a caracterização dos genes de Glutationa Peroxidase (*GPX*) em arroz *(Oryza sativa*) e Arabidopsis (*Arabidopsis thaliana*). Assim como a análise funcional das GPX localizadas na mitocrôndria (*OsGPX1* e *OsGPX3*) e nos cloroplastos (*OsGPX4*) no desenvolvimento de plantas de arroz, e a participação das AtGPXs na determinação da arquitetura da raiz dependente de hormônios em Arabidopsis.

Objetivos específicos

- Analisar o padrão de expressão dos genes de *GPXs* (*OsGPX1*-*OsGPX5*) em plantas de arroz submetidas a diferentes tipos de estresses;
- Determinar o perfil de expressão dos genes de GPXs durante o desenvolvimento vegetativo e reprodutivo;
- Determinar a localização subcelular das diferentes isoformas de GPX em protoplastos de arroz;
- Obter plantas de arroz silenciadas para os genes de GPX;
- Analisar os efeitos do silenciamento em plantas de arroz transgênicas;
- Estudar o perfil de expressão pela avaliação da atividade do gene *Gus* dirigido pela região promotora dos genes de GPX em arroz;
- Identificar mutantes de T-DNA para os genes de GPX de *Arabidopsis thaliana* (AtGPX1-AtGPX8);
- Analisar fenotipicamente os mutantes de *AtGPX* comparados à planta tipo selvagem;
- Estudar o perfil de expressão dos genes de AtGPX em condições normais de crescimento e após o tratamento com os hormônios auxina e strigolactona em plantas de Arabidopsis tipo selvagem;
- Avaliar o papel de AtGPX na determinação da arquitetura da raiz dependente da resposta aos hormônios auxina e strigolactona.

CAPÍTULO 1

Genes de Glutationa Peroxidase (*GPX*) de *Arabidopsis thaliana*

Neste capítulo serão apresentados os resultados referentes aos estudos de caracterização dos genes de Glutationa Peroxidase de *Arabidopsis thaliana*.

Este trabalho foi desenvolvido durante o período de doutorado sanduíche (CAPES – Ciência sem Fronteiras) de Fevereiro a Setembro de 2012 na Universidade de Leeds, Reino Unido, sob a orientação da professora Christine Helen Foyer.

Neste trabalho foi demonstrado que diferentes genes de *GPX* de Arabidopsis possuem funções específicas no controle hormonal da arquitetura da raiz. Foram estudados sete mutantes por inserção de T-DNA dentre os oito da família *GPX* de Arabidopsis. Pequenas diferenças fenotípicas foram observadas na parte aérea e raízes de alguns dos mutantes quando comparados ao tipo selvagem. Pelo menos três genes de *GPX (GPX2, GPX3* e *GPX6*) são requeridos no controle hormonal, via auxina, strigolactona e ácido abscísico, da arquitetura da raiz de *Arabidopsis thaliana.*

Este estudo resultou em um artigo intitulado: "Specific roles for glutathione peroxidases (*AtGPX*) in the hormone-dependent control of root architecture", a ser re-submetido para publicação no periódico *Plant Physiology*. O artigo é apresentado a seguir.

Running head: Glutathione Peroxidases and root architecture

Corresponding author: Christine H. Foyer
Centre of Plant Science
Research Institute of Integrative and Comparative Biology
Faculty of Biological Science University of Leeds
Leeds
LS2 9JT
UK

Tel +44 (0)113 343 1421; Fax +44(0)113 323 3144

Email: c.foyer@leeds.ac.uk

Research area: Signal Transduction and Hormone Action

Specific roles for *Arabidopsis* glutathione peroxidases (AtGPX) in the hormone-dependent control of root architecture

Gisele Passaia[1,2], Guillaume Queval[1], Marcia Margis-Pinheiro[2] and Christine H. Foyer[1]

[1] Centre for Plant Sciences, Faculty of Biology, University of Leeds, Leeds, LS2 9JT, UK

[2] Depto. Genética, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500. Prédio 43.312, CEP 91501-970 Porto Alegre, RS, Brazil

Footnotes: This work was funded in part by an EU Marie Curie ITN (PITN-GA-2008-215174: Chloroplast Signals, for DS) and in part by a Marie Curie individual fellowship grant to GQ (PIEF-GA-2009-252927: ROXNP). Gisele Passaia was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq.

Author to whom correspondence should be addressed: Email: c.foyer@leeds.ac.uk
Tel +44 (0)113 343 1421; Fax +44(0)113 323 3144

The author(s) responsible for distribution of materials integral to the findings presented in this article in accordance with policy described in the instructions for Authors (www.plantphysiol.org) is: Christine H. Foyer (c.foyer@leeds.ac.uk).

ABSTRACT

The roles of *Arabidopsis thaliana* glutathione peroxidases (AtGPX) in the regulation of root architecture were investigated using T-DNA insertion mutants (*gpx1*, *gpx2*, *gpx3*, *gpx4*, *gpx6*, *gpx7* and *gpx8*). The mutants showed few phenotypic changes relative to the wild type and treatment with auxin increased the number of lateral roots in all genotypes. However, the auxin-dependent increase in lateral root proliferation was greater in the *gpx2* mutants than controls. Auxin-dependent stimulation in the number of early stage (I-II) primordia was absent from the *gpx2* mutants. The inhibitory action of auxin on primary root growth was greater in the *gpx1* and *gpx7* mutants than the wild type, but there were no differences in lateral root production in these genotypes. The addition of the artificial strigolactone GR24 decreased the length of the primary roots and the number of lateral roots in all genotypes. However, lateral root production was much less sensitive to GR24 in the *gpx3* and *gpx4* mutants than wild type controls but it was more sensitive in the *gpx2* mutants. The GR24-dependent inhibition of lateral root development occurred after emergence and in stage I-II of LRP in the wild type. In contrast, GR24 increased the number of stage I-II of LRP in the *gpx2* and *gpx3* mutants. While abscisic acid (ABA)-dependent inhibition of lateral root formation was less marked in the *gpx2* mutants, the *gpx6* mutants showed a higher sensitivity to ABA than the wild type. These results suggest that specific AtGPXs are required for the hormone-dependent control of root architecture.

INTRODUCTION

Glutathione (GSH) peroxidases (GPXs: EC 1.11.1.9 and EC1.11.1.12) belong to a widespread family of non-haeme thiol peroxidases, which catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Herbette et al., 2007). The active site is composed of a catalytic triad formed by selenocysteine /cysteine, glutamine and tryptophan. However, while animal GPXs are selenoproteins that contain a selenocysteine at the catalytic site the plant enzymes do not bind selenium (Herbette et al., 2007; Toppo et al., 2008). Moreover, the animal GPXs exclusively use GSH as the reducing substrate. In contrast, recent evidence suggests that a large number of plant GPXs use thioredoxin and they are therefore are better described as thiol peroxidases (Navrot et al., 2006).

In addition to their role in preventing uncontrolled oxidation, GPXs can interact with other proteins and hence they are also considered to have signaling functions (Delaunay et al., 2002; Miao et al., 2006). Some members of the GPX superfamily can form tetrameric structures with other proteins in response to oxidants such as H_2O_2 or hydroperoxides. In yeast, for example, GPX3 is considered to activate the Yeast Activation Protein 1 (Yap-1) transcription factor via an oxidation-induced event in order to promote the activation of genes encoding thiol-reducing and antioxidant proteins (Delaunay et al., 2002). In *Arabidopsis thaliana*, the AtGPX3 protein physically interacts with protein phosphatase type 2C (PP2C) proteins such as ABSCISIC ACID INSENSTIVE (ABI) 1 and ABI2, in order to activate plasma membrane Ca_2^+ and K^+ channels that facilitate stomatal closure (Miao et al., 2006). Two stromal GPXs (AtGPX1 and AtGPX7) were shown to be important in chloroplast functions, particularly light acclimation and also in plant immune responses (Chang et al., 2009).

Arabidopsis contains a small GPX gene family comprising eight members. Based on phylogenetic studies, the AtGPX proteins have been assigned to cytosol (AtGPX4 and AtGPX5), endoplasmic reticulum/cytosol (AtGPX2 and AtGPX3), mitochondria (AtGPX6), chloroplasts (AtGPX1 and AtGPX7) or the cytosol/apoplast (AtGPX8) (Margis et al., 2008). Recently, AtGPX8 was localized in the nucleus as well as the cytosol of onion and *Arabidopsis* (Gaber et al., 2012). Moreover, the roots of *gpx8 Arabidopsis* mutants showed a greater sensitivity to the oxidative stress induced by paraquat than the wild type controls (Gaber et al., 2012). Interestingly, the *gpx8*

mutants showed enhanced DNA damage suggesting a role for this protein in the protection of the nucleus. These studies demonstrate that GPXs fulfill important functions in leaves and roots but the precise roles of each GPX form in plant biology particularly in the control of development, remains largely uncharacterized.

The plant hormone auxin is a crucial signal for the initiation of lateral root development. Initially, auxin triggers the first divisions of lateral root founder cells in the pericycle tissue of the primary root (Casimiro et al., 2003). This is facilitated by the accumulation of auxin firstly in the central cells of the lateral root primordium (LRP) and later at the tip of the LRP. Auxin is considered to regulate at least three steps in LPR development: initiation, LRP establishment and the activation of LR meristem (De Smet et al., 2003). Auxin accumulation promotes the degradation of Aux/IAA proteins, enabling auxin response factor (ARF) proteins to transcribe genes required to pattern the new LRP (Péret et al., 2009). Leaf-derived auxin also plays an important role in the further development of the lateral root after emergence (Bhalerao et al., 2002).

The action of auxin in the control of LRP development is modulated by a second group of hormones called strigolactones (SLs) which influence auxin signalling pathways on multiple levels (Agusti et al., 2011). In addition to inhibiting shoot branching SLs inhibit lateral root initiation, while also acting as positive regulators of root-hair elongation (Kapulnik et al., 2011). SL synthesis, which is stimulated by auxin, blocks auxin transport (Bennett et al., 2006; Crawford et al., 2010; Balla et al., 2011). The abundance of PIN family of auxin-efflux carrier proteins and auxin transport are enhanced in the stems of *max1-1* mutants that are defective in SL signalling. Moreover, the treatment with the synthetic strigolactone GR24 decreases the abundance of the PIN1 protein and represses auxin transport (Bennett et al., 2006; Crawford et al., 2010).

Like SLs, abscisic acid (ABA) is an inhibitor of lateral root development. ABA not only suppresses the auxin response in the LRP but it also regulates LRP development in an auxin-independent manner (De Smet et al., 2003). For example, the *abscisic acid insentive 4* (*abi4*) mutant, which is insensitive to ABA, has an increased number of lateral roots relative to the wild type (Shkolnik-Inbar and Bar-Zvi, 2010). Moreover, the expression of PIN1, which is enhanced in *abi4* mutants, is less sensitive to ABA-mediated inhibition than in the wild type. The ABI4 transcription factor may therefore mediate ABA-dependent inhibition of lateral root formation by reducing polar auxin transport (Shkolnik-Inbar and Bar-Zvi, 2010).

NADP-linked thioredoxin and glutathione systems have previously been shown to be required for auxin transport and signaling in *Arabidopsis* (Bashandy et al., 2010). Moreover, the auxin-induced expression of glutathione S-transferases (GST) such as AtGSTF2 has long been recognised. AtGSTF2 binds indole-3-acetic acid (IAA) and the auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA), a function that may be important in stress-mediated growth responses (Smith et al., 2003). Another auxin-inducible GST, *AtGSTU17* fulfils important roles in seedling development. The *atgstu17* mutants are less sensitive to auxin and have lower numbers of lateral roots in the presence of auxin. They are also less sensitive to ABA-mediated inhibition of root development (Jiang et al., 2010). The roles of other thioredoxin-dependent or glutathione-related proteins such as GPXs in the hormone-dependent control of root development has not been reported to date. The present study was therefore undertaken to explore the roles of the different AtGPX forms in the action of auxin, SL and ABA in the control of root development in *Arabidopsis*.

RESULTS

Specific changes in the abundance of *AtGPX* mRNAs in the *gpx1, gpx2, gpx3, gpx4, gpx6, gpx7* and *gpx8* mutants relative to the wild type.

The relative expression of the different *AtGPX* genes was compared in the roots and shoots in Col-0 or Col-3 wild type (Fig. 1A). *AtGPX1* and *AtGPX2* were highly expressed in shoots. *AtGPX2* was also highly expressed in roots, as was *AtGPX6* (Fig. 1B). *AtGPX3* was more highly expressed in the shoots of mutants lacking *gpx1*, *gpx2*, *gpx4, gpx6, gpx7* and *gpx8* while *AtGPX8* were more highly expressed in the shoots of mutants lacking *gpx1, gpx2, gpx3, gpx6 and gpx7* whereas *AtGPX3, AtGPX5* and *AtGPX8* were more highly expressed in the shoots of the *gpx2* mutants (Fig. 1B). *AtGPX4* was expressed at very low levels in both roots and shoots of the wild type plants (Fig. 1A) but while its expression was unchanged in the shoots of all the mutants analyzed, *AtGPX4* expression was decreased in the roots of all the mutant lines except g*px1* and *gpx3*. *AtGPX5* was more highly expressed in the shoots of the *gpx2, gpx6, gpx7* and *gpx8* mutants but reduced in *gpx1* shoots. However, *AtGPX2* transcripts were less abundant in the roots of the *gpx7* mutants while *AtGPX7* transcripts were less abundant in the roots of the *gpx8* and *gpx3* mutants but more highly expressed in shoots of *gpx4* mutant. Surprinsingly, *AtGPX7* transcripts were up-regulated only in the roots of *gpx7*, this could be explained by the location of the T-DNA insertion, acting as a promoter enhancer in this tissue instead of knocking-out the gene. The fold change of gene expression for each mutant compared to wild-type is shown in Supplemental Fig. 1.

Phenotype differences in the *gpx1, gpx2, gpx3, gpx4, gpx6, gpx7* and *gpx8* shoots and roots relative to controls

The shoot phenotypes of *gpx1*, gpx2, gpx3, *gpx4, gpx6, gpx7* and *gpx8* mutants was similar to that of the respective wild types when plants were grown in soil for 4 weeks. No significant differences in shoot biomass (fresh weight) or pigment (chlorophylls or carotene) contents were observed in any of the lines (data not shown). However, the *gpx2* and *gpx8* mutants had significantly fewer leaves than the wild type, while the *gpx7* mutants significantly more leaves than the wild type rosettes.

The root phenotypes were similar in the *gpx1*, *gpx3, gpx4, gpx6, gpx7* and *gpx8* mutants to the respective wild types when seedlings were grown on plates for 5 days (Fig. 2A). However, the length of the primary root was a little longer in the *gpx4* mutants, which also had more lateral roots than the wild type at this point (Fig. 2A,C). Moreover, *gpx2* mutants had fewer lateral roots than the wild type (Fig. 2C).

Strigolactone (GR24)-dependent inhibition of lateral root production was increased in the *gpx2* mutants but decreased in the *gpx3* and *gpx4* mutants

The root phenotypes were similar in all genotypes in the absence of hormone treatment, except that the *gpx2* mutants had significant fewer lateral roots that the wild type controls (Fig. 2C). The addition of the artificial strigolactone GR24 led to a reduction in the length of the primary root and in the number of lateral roots relative to untreated controls (Fig. 2C, D). The response to GR24 was similar in *gpx1*, *gpx6. gpx7* and *gpx8* mutants to that observed in wild type seedlings. However, lateral root production was more inhibited in the *gpx2* mutants. In contrast, The GR24-dependent inhibition of lateral root production was significantly decreased in the *gpx3* and *gpx4* mutants relative to their respective wild type controls (Fig. 2D).

Auxin (IAA)-dependent stimulation in root architecture was more marked in the *gpx2* mutants

The root phenotypes were similar in all genotypes in the absence of auxin, except that the *gpx2* and *gpx3* mutants had significantly fewer lateral roots relative to their respective wild type controls (Fig. 3). The addition of auxin led to a significant reduction in the length of the primary root in all genotypes (Fig. 3B) and the number of lateral roots was increased relative to controls (Fig. 3D). However, the auxin-induced increase in the number of lateral roots was significantly higher in the *gpx2* mutants. Moreover, the auxin-induced increase in the number of lateral roots was significantly lower in the *gpx6* mutants (Fig. 3D) relative to controls (Fig. 3C).

Strigolactone-dependent decreases in the number of stage I-II and emerged stage primordia were absent from the *gpx2* and *gpx3* mutants

A more-detailed analysis of the different stages of the development of the LRP that are here grouped as stage I-II, III-V, VI-VII and emerged stage primordial (Malamy and Benfey, 1997; Péret et al., 2009), revealed that there were few statistically-significant

genotype-dependent differences in the early stages of LRP development in the absence of GR24 (Fig. 4) or auxin (Fig. 5), except that the *gpx2* mutants showed a significant decrease in stage I-II primordia in the absence of strigolactone (Fig. 4B, D).

Treatment with GR24 led to a significant decrease in the number of stage I-II and emerged stage primordia in the Col-0 and Col-3 wild types relative to controls (Fig. 4A, C). In marked contrast, the treatment with strigolactone significantly increased the number of stage I-II primordia in the *gpx2* and *gpx3* mutants, which also had a greater total number of lateral roots relative to wild-type controls after GR24 treatment (Fig. 4B, D).

The *gpx2* mutants do not show auxin-dependent increases in the numbers of stage I-II primordia

Auxin treatment caused a significant increase in the number of stage I-II, III-IV and emerged stage primordia in the Col-0 and Col-3 wild type controls (Fig. 5A, C). However, the auxin-dependent stimulation of the stage I-II primordia was absent in the *gpx2* mutants (Fig. 5B). In contrast, a significant increase in the auxin-dependent stimulation of the numbers of stages I-II primoria was observed in the *gpx3* mutants relative to the wild type controls (Fig. 5D).

Strigolactone-dependent and auxin-dependent effects on the expression of *AtGPX* genes in roots and shoots

The addition of GR24 had little effect on the expression of the different *AtGPX* genes, except that the abundance of *AtGPX1* transcripts was increased in both shoots and roots after strigolactone treatment (Fig. 6A). Similarly, *GPX2* and *GPX7* transcripts were more abundant in roots but not in shoots following GR24 treatment (Fig. 6A). In contrast, auxin induced the expression all the *AtGPX* genes in the shoots except *AtGPX1* and *AtGPX7*, which higher following auxin treatment (Fig. 6B). Moreover, while *AtGPX1* and *AtGPX2* transcripts were lower in the roots of auxin treated seedlings, *AtGPX4* and *AtGPX7* mRNAs were higher in the roots after auxin treatment (Fig. 6B).

Abscisic acid-dependent decreases in emerged stage primordia were absent from the *gpx2* and *gpx3* mutants

Treatment with increasing concentrations of ABA, led to a progressive decrease in the number of lateral roots in the wild type seedlings, together with a small effect on primary root growth (Fig. 7A, B). As in other experiments (e.g. Fig. 3), the *gpx2* mutants had fewer lateral roots than the wild type in the absence of added hormone. The ABA-induced inhibition in lateral root formation was less marked in the *gpx2* mutants relative to the wild type controls (Fig. 7B). However, lateral root production was much more sensitive to ABA-induced inhibition in the *gpx6* mutants than in the respective wild type (Fig. 7).

DISCUSSION

The interplay between different hormones such as auxin, strigolactones and ABA plays a key role in the control of root development and its regulation by metabolic cues and environmental triggers. The evidence presented here demonstrates that several AtGPX forms are required for correct hormone-dependent responses in root development. For example, the auxin-dependent stimulation of stage I was absent in the *gpx2* mutants. This would suggest that the *gpx2* mutants are impaired in processes that occur at the earliest stages of LRP development. This would suggest that AtGPX2 is required for the earliest stage of LRP development. Other mutants showing a disruption in lateral root emergence have been shown to be defective in auxin transport from shoots to roots. For example, the *lax3* mutants show an increased proportion of stage I primordia in the roots. The *LAX3* gene encodes a high affinity auxin influx carrier, which is required for the development and emergence of lateral roots (Swarup et al., 2008). The delay in the developmental progression of *lax3* mutant lateral roots, in which the majority of primordium are found at stage I is therefore caused by a deficit in leaf-derived auxin (Bhalerao et al., 2002). However, the *gpx2* mutants do not show an increase at stage I primordial either in the absence or presence of auxin. Moreover, the GR24-dependent inhibition of lateral root formation was increased in the *gpx2* mutants, indicating that they remain sensitive to strigolactone-dependent inhibition of auxin transport. Conversely, the *gpx3* mutants have a decreased sensitivity to strigolactone, which may suggest that auxin transport is already impaired as a result of the loss of AtGPX3 function.

The *gpx2* mutants are also less sensitive to ABA-dependent inhibition of lateral root branching relative to the wild type. Additionally, *gpx6* mutants were more sensitive to ABA-induced inhibition relative to the wild type controls, as well as being more sensitive to auxin-dependent stimulation of lateral root production. The *gpx2* root phenotype resemble that described for a mutant defective in GSTU17, which also showed alterations in auxin-dependent regulation of lateral roots and an insensitivity to ABA (Jiang et al., 2010). In the case of GSTU17, these effects were linked to changes in glutathione, which is known to influence auxin transport (Bashandy et al, 2010). However, given that the plant GPXs have a higher affinity for thioredoxin than GSH

(Herbette et al., 2007; Toppo et al., 2008), it is likely that thioredoxin rather than GSH has a role in the auxin and ABA-mediated processes observed in the *gpx2* mutant. Like GSH, thioredoxin is considered to be important in the auxin-dependent control of root architecture (Bashandy et al, 2010). For example, the *Arabidopsis ntrc* mutants that lack NADPH-thioredoxin reductase C have lower auxin contents than the wild type tissues and they show impaired root growth and lateral root formation (Kirchsteiger et al., 2012). Interestingly, the lateral root phenotype was rescued by expression of *NTRC* in leaves but not in roots (Kirchsteiger et al., 2012). These studies linked the redox state of the chloroplasts to the lateral root phenotype. AtGPX1 and AtGPX7 fulfil important roles in chloroplasts (Chang et al., 2009)**.** However, the data presented here show that the effect of auxin on the development of lateral roots was not modified in the *gpx1* and *gpx7* mutants.

Thioredoxins can modulate other functions in roots that have a direct impact on lateral root production. For example, the *gat1* mutant (gfp-arrested trafficking), which is defective in thioredoxin m3, accumulates high levels of callose leading to the occlusion of the plasmodesmata in the root meristem resulting in arrested root development (Benitez-Alfonso et al., 2009). We have not measured callose production in any of the *gpx* mutants studied here but they do not show the extreme inhibition of lateral root growth observed in the *gat1* mutants.

The data presented here would suggest that several steps in the hormone-mediated control of lateral root formation in *Arabidopsis thaliana* require AtGPXs. The *gpx2* and *gpx3* mutants show a markedly different response to the addition of strigolactone than the respective wild type controls, particularly in terms of the numbers of stage I-II primordia, showing that the classic strigolactone response is absent in the mutants. The lack of strigolactone-dependent repression in lateral root development in the *gpx3* mutants would suggest that auxin transport is already inhibited in these mutants in the absence of added strigolactone, as illustrated in Fig.8. In contrast, while the *gpx2* mutants have lower numbers of stage I-II primordia relative to the wild type controls, they remain sensitive to strigolactones, suggesting that the site of action of AtGPX2 in the hormone-dependent response is different from that of AtGPX3, as illustrated in Fig.8. The *gpx2* and *gpx6* mutants show different alterations in their responses to auxin and ABA, suggesting that AtGPX2 and AtGPX6 are required for auxin-dependent regulation of LRP development in different ways, but that their sites of action are more downstream of auxin transport than AtGPX3, as illustrated in Figure 8.

Taken together, these data provide evidence that several AtGPX forms participate in the fine-tuning of hormone-dependent responses that control root development by redox-dependent processes and triggers.

MATERIALS AND METHODS

Identification and isolation of T-DNA insertion mutants

Arabidopsis T-DNA insertion lines from SAIL (Sessions et al., 2002), SALK (Alonso et al., 2003) and WiscDsLox (Woody et al., 2007) collections were obtained. Donor stock numbers SALK_027373 (*gpx1* – At2g25080), SALK_082445 (*gpx2* – At2g31570), SAIL_278_E06 (*gpx3* – At2g43350), SALK_139870 (*gpx4* – At2g48150), WISCDSLOX_321_H10 (*gpx6* – At4g11600), SALK_023283 (*gpx7* – At4g31870), SALK_127691 (*gpx8* – At1g63460) were obtained from ABRC (Ohio State University) and NASC (Nottingham Arabidopsis Stock Center). All T-DNA mutants are in Col-0 background, except for *gpx3* mutant, which is in Col-3 background. Plants were germinated and grown in conventional soil and to confirm the homozygozity of the insertion, PCR was performed with genomic DNA using gene specific oligonucleotides Left border specific primers for T-DNA sequences:

SALK_LB: 5'-TGGACCGCTTGCTGCAACTCTC-3',

SAIL_LB: 5'-AAATGGATAAATAGCCTTGCTTCC-3',

WISC_LB: 5'-AACGTCCGCAATGTGTTATTAAGTTGTC-3';

AtGPX1F: 5'-TGAAAATTCCTCGTGTTTTGG-3';

AtGPX1R: 5'-TTTTCAAGTGTTCAAAGCGAAG-3';

AtGPX2F: 5'-TTGTTTGCCACACATTGATTG-3';

AtGPX2R: 5'-TTTTACCATTTGGAAGCAACG-3';

AtGPX3F: 5'-TTCAGTCGAAGAAGCAAACG-3';

AtGpx3R: 5'-AACAGTTGCCAAACTCTTAGGG-3';

AtGPX4F: 5'-GCAAGCTCATCTCCTTTGTTG-3';

AtGPX4R: 5'-AAAAATGCCTGTCATGCATG-3';

AtGPX6F: 5'-CCATTGAGAAGCAACGTTCAC-3';

AtGPX6R: 5'-TGGCCTCCTTTCTTTCTTTTG-3';

AtGPX7F: 5’-AAGGGCTGCAGCAGAGAAG-3’;

AtGPX7R: 5’-TTGAACCGATCGAAAATTCC-3’;

AtGPX8F: 5’-CAGTGGGATGACAAACTCAAAC-3’;

AtGPX8R: 5’-CAGAACCACCCCACTTTCAG-3’.

The position of the T-DNA insertion sequence for each genotype is shown in Supplemental Figure 2.

Growth of plants and hormone treatments

Arabidopsis seeds of wild type (Col-0 and Col-3) and *gpx1*, *gpx2*, *gpx3*, *gpx4*, gpx6, *gpx7* and *gpx8* mutant were surface sterilized two minutes in 75% ethanol, five minutes in 4% sodium hypochlorite and several washes in sterilized water until the pH is around 7. All the experiments were performed in vertical plates containing half-strength Murashige and Skoog medium (½ MS medium, pH 5.7) with 0.01% myo-inositol, 0.05% MES, 1% sucrose and 0.8% plant agar on 12 cm square plates and stored for two days in cold and dark to synchronize germination. After that period, the plates were placed on a plant growth cabinet with 16 hours day photoperiod and 22°C. After three days the seedlings were gently transferred using forceps to new plates containing the same medium plus 2 µM of GR24 and grown for a further 5 days. NAA (1-naphthalene acetic acid) assay were performed by placing the seeds on ½ MS vertical plates for 5 days atlight and then seedlings were transferred to ½ MS medium containing 1 µM NAA for 3 days. ABA (Sigma) was added to the media at 0.3 µM or 0.5 µM concentrations. After 3 days in dark and cold, plates containing seeds and ABA were transferred to a plant growth cabinet with the same conditions described above for 8 days.

Root architecture and rosette measurements

The root length and number of lateral roots formed per treatment were analyzed on 8 days old seedlings using ImageJ software (http://rsbweb.nih.gov/ij/).

Gene expression analysis

RNA was extracted from the shoots and roots of 10 day old Col-0, Col-3, *gpx1*, *gpx2*, *gpx3*, *gpx4*, *gpx6*, *gpx7* and *gpx8* plantlets grown in ½ MS medium in a growth chamber at 20 °C with a 16 h photoperiod using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For each genotype, three biological replicates for the shoot and the root were prepared. Reverse transcription of 1 µg RNA into cDNA was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The qPCR was performed on 20 ng cDNA with the QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany) in the presence of 0.5 µM primers in a CFX96 thermocycler (Biorad, Hercules, California, USA). PCR conditions were as follows: 5 min 95°C, 45 cycles 10 sec 95°C and 30 sec 60°C. Additionally melting curve analysis was performed at the end of each run to ensure specificity of the products. The mean value of three replicates was normalized using *Actin2* as internal control (primer sequences can be found in Supplemental Table 1). Quality of amplification curves was checked with the LinRegPCR software (Ramakers et al., 2003) and curves with R above 0.995 and efficiencies between 1.9 and 2.1 were kept for following expression value calculations. All expression data analyses were performed after comparative quantification of amplified products by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

Root stageing

Measurements of the stages of lateral root development were performed as previously described (Malamy and Benfey, 1997). Roots were incubated in 0.24 N HCl and 20% methanol at 62°C for 20 minutes. This solution was then replaced with 7% NaOH with 60% ethanol and the roots were then incubated for further15 minutes at room temperature. Following rehydration in 40% ethanol, followed by 20% ethanol and then 10% ethanol, the roots were infiltrated for 15 minutes in 5% ethanol with 25% glycerol. The root samples were then maintained in 50% glycerol until microscopic analysis (M 4000-D, Swift). The stages of primordium development were classified as described by Péret et al. (2009) as follows: stage I (single layered primordium composed of up to ten small cells of equal length formed from individual or a pairs of pericycle founder cells), stage II (periclinal cell division forming an inner and an outer layer), stages III, IV, V, VI and VII (anticlinal and periclinal divisions create a dome-shaped primordium), stage VIII (emergence of the primordium from the parental root).

Statistical analysis

Data represent mean ± standard error of mean (SEM). Statistical analysis was performed by Student t-test. The values were considered statically different when $p < 0.05$.

ACKNOWLEDGMENT

Guillaume Queval is funded by Marie Curie Individual Fellowship: PIEF-GA-2009-252927: ROXNP. The authors thank the Salk Institute, Syngenta and the University of Wisconsin for the production of the T-DNA insertion lines and the Ohio State University and the University of Nottingham for the distribution of the seeds. We are grateful to Barbara Karpinska and Belen Marquez-Garcia for useful discussions.

References

Agusti J, Herold S, Schwarz M, Sanchez P, Ljung K, Dun EA, Brewer PB, Beveridge CA, Sieberer T, Sehr EM, et al (2011) Strigolactone signaling is required for auxin-dependent stimulation of secondary growth in plants. Proc Natl Acad Sci USA **108:** 20242–20247

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653–657

Balla J, Kalousek P, Reinöhl V, Friml J, Procházka S (2011) Competitive canalization of PIN-dependent auxin flow from axillary buds controls pea bud outgrowth. Plant J **65:** 571–577

Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld J-P (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. Plant Cell **22:** 376–391

Benitez-Alfonso Y, Cilia M, San Roman A, Thomas C, Maule A, Hearn S, Jackson D (2009) Control of Arabidopsis meristem development by thioredoxin-dependent regulation of intercellular transport. Proc Natl Acad Sci USA **106:** 3615–3620

Bennett T, Sieberer T, Willett B, Booker J, Luschnig C, Leyser O (2006) The Arabidopsis MAX pathway controls shoot branching by regulating auxin transport. Curr Biol **16:** 553–563

Bhalerao RP, Eklöf J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in Arabidopsis seedlings. Plant J **29:** 325–332

Casimiro I, Beeckman T, Graham N, Bhalerao R, Zhang H, Casero P, Sandberg G, Bennett MJ (2003) Dissecting Arabidopsis lateral root development. Trends Plant Sci **8:** 165–171

Chang CCC, Slesak I, Jordá L, Sotnikov A, Melzer M, Miszalski Z, Mullineaux PM, Parker JE, Karpinska B, Karpinski S (2009) Arabidopsis chloroplastic glutathione peroxidases play a role in cross talk between photooxidative stress and immune responses. Plant Physiol **150:** 670–683

Crawford S, Shinohara N, Sieberer T, Williamson L, George G, Hepworth J, Müller D, Domagalska MA, Leyser O (2010) Strigolactones enhance competition between shoot branches by dampening auxin transport. Development **137:** 2905–2913

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111:** 471–481

Gaber A, Ogata T, Maruta T, Yoshimura K, Tamoi M, Shigeoka S (2012) The involvement of Arabidopsis glutathione peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. Plant Cell Physiol **53:** 1596–1606

Herbette S, Roeckel-Drevet P, Drevet JR (2007) Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. FEBS J **274:** 2163–2180

Jiang HW, Liu MJ, Chen IC, Huang CH, Chao LY, Hsieh HL (2010) A glutathione S-transferase regulated by light and hormones participates in the modulation of Arabidopsis seedling development. Plant Physiol **154:** 1646–1658

Kapulnik Y, Delaux PM, Resnick N, Mayzlish-Gati E, Wininger S, Bhattacharya C, Séjalon-Delmas N, Combier JP, Bécard G, Belausov E, et al (2011) Strigolactones affect lateral root formation and root-hair elongation in Arabidopsis. Planta **233:** 209–216

Kirchsteiger K, Ferrández J, Pascual MB, González M, Cejudo FJ (2012) NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in Arabidopsis. Plant Cell **24:** 1534–1548

Lichtenthaler HK (1987) ChlorolShylls and Carotenoids: Pigments of Photosynthetic Biomembranes. Methods Enzymol **148:** 350–382

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:** 402–408

Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of Arabidopsis thaliana. Development **124:** 33–44

Margis R, Dunand C, Teixeira FK, Margis-Pinheiro M (2008) Glutathione peroxidase family - an evolutionary overview. FEBS J **275:** 3959–3970

Miao Y, Lv D, Wang P, Wang XC, Chen J, Miao C, Song CP (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18:** 2749–2766

Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, Rey P, Knaff DB, Issakidis E, Jacquot JP, Routhier N (2006) Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol **142:** 1364–1379

Péret B, De Rybel B, Casimiro I, Benková E, Swarup R, Laplaze L, Beeckman T, Bennett MJ (2009) Arabidopsis lateral root development: an emerging story. Trends Plant Sci **14:** 399–408

Ramakers C, Ruijter JM, Deprez RHL, Moorman AF. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett **339:** 62–66

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nat Protoc **3:** 1101–1108

Sessions A, Burke E, Presting G, Aux G, Mcelver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, et al (2002) A High-Throughput Arabidopsis Reverse Genetics System. Plant Cell **14:** 2985–2994

Shkolnik-Inbar D, Bar-Zvi D (2010) ABI4 mediates abscisic acid and cytokinin inhibition of lateral root formation by reducing polar auxin transport in Arabidopsis. Plant Cell **22:** 3560–3573

Smet I, Signora L, Beeckman T, Inzé D, Foyer CH, Zhang H (2003) An abscisic acid-sensitive checkpoint in lateral root development of Arabidopsis. Plant J **33:** 543–555

Smith AP, Nourizadeh SD, Peer W A., Xu J, Bandyopadhyay A, Murphy AS, Goldsbrough PB (2003) Arabidopsis AtGSTF2 is regulated by ethylene and auxin, and encodes a glutathione S-transferase that interacts with flavonoids. Plant J **36:** 433–442

Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, et al (2008) The auxin influx carrier LAX3 promotes lateral root emergence. Nat Cell Biol **10:** 946–954

Toppo S, Vanin S, Bosello V, Tosatto SCE (2008) Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. Antioxid Redox Signal **10:** 1501–1514

Woody ST, Austin-Phillips S, Amasino RM, Krysan PJ (2007) The WiscDsLox T-DNA collection: an arabidopsis community resource generated by using an improved high-throughput T-DNA sequencing pipeline. J Plant Res **120:** 157-65

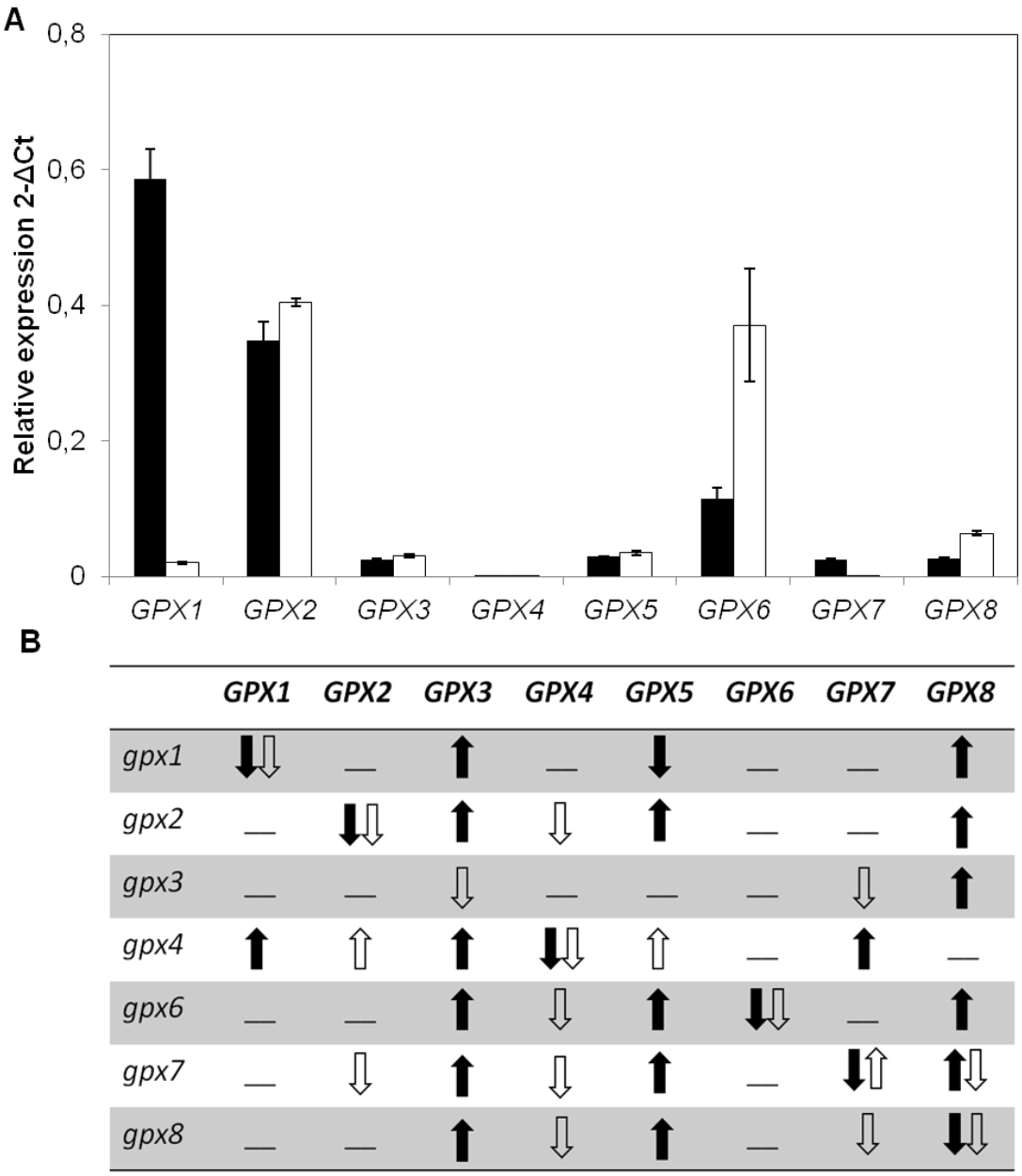


Figure 1. The relative expression of *GPX* genes in Col-0 shoots (black bars) and roots (white bars) (A) and in the different GPX mutant backgrounds (B). Data are the mean ± SE of three biological replications.

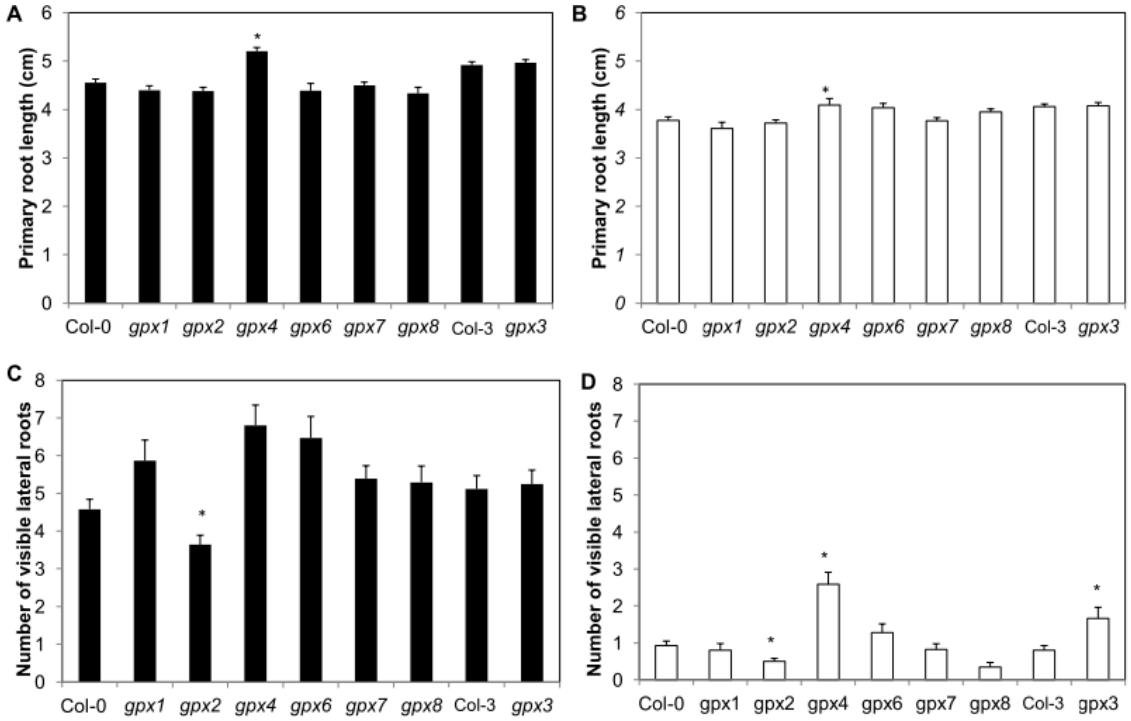


Figure 2. A comparison of root phenotypes and the effects of the strigolactone GR24 on root architecture in the different genotypes. The length of the primary roots (A, B) and number of visible lateral roots (C, D) was measured in the *gpx* mutant lines relative to the respective wild types (Col-0 and Col-3), together with the effect of strigolactones (GR24) on these parameters. Black and white bars denote control and 2µM GR24 conditions, respectively. Data are the mean ± SE of one to three biological replications. * Asterisks indicate significant differences $p<0.05$.

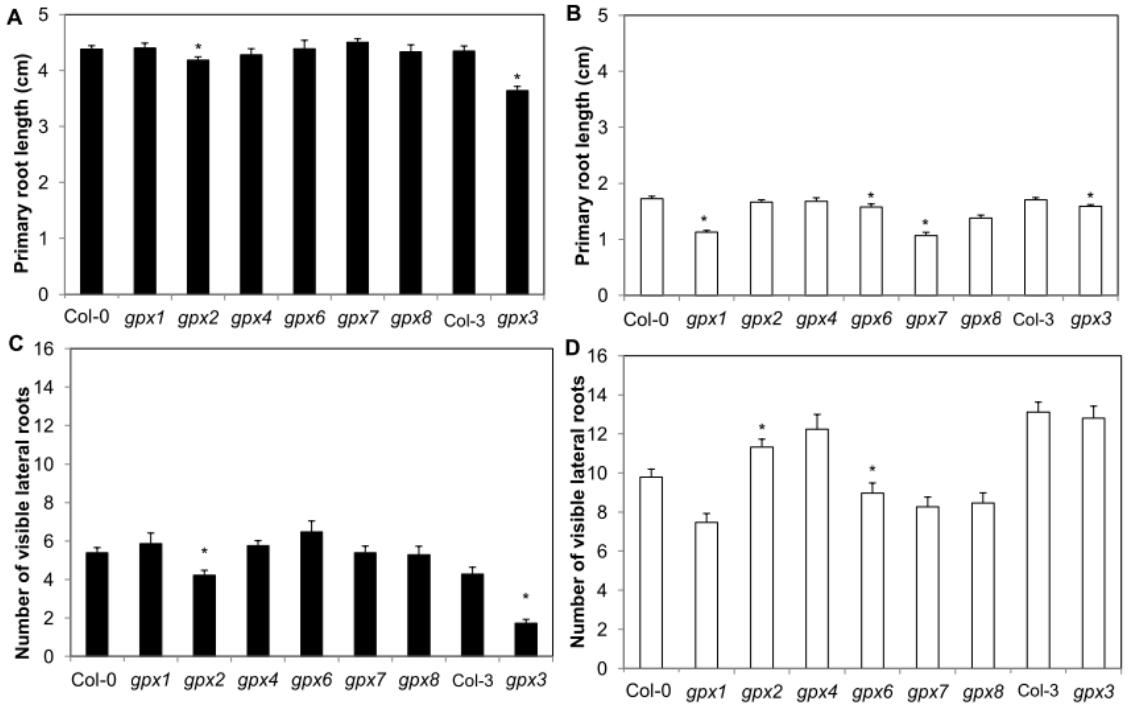


Figure 3. The effects of the auxin (IAA) on root architecture in the different genotypes. The length of the primary roots (A, B) and number of lateral roots (C, D) was measured in the *gpx* mutant lines relative to the respective wild types (Col-0 and Col-3), together with the effect of auxin on these parameters. Black and white bars denote control and 1µM NAA conditions, respectively. Data are the mean ± SE of one to three biological replications. * Asterisks indicate significant differences $p<0.05$ between mutant and wild-type.

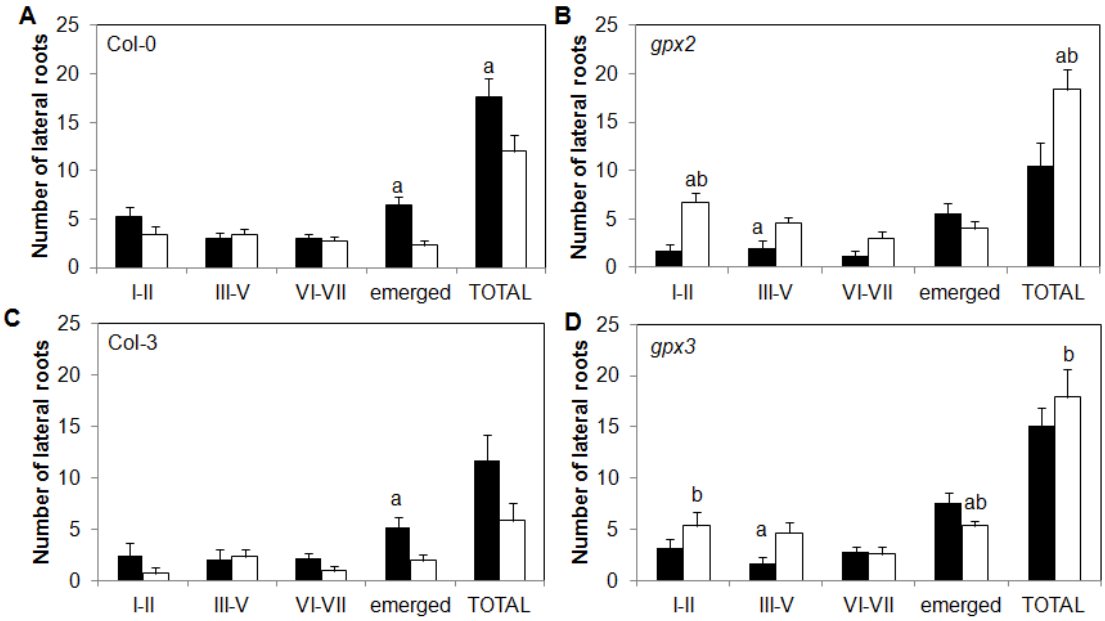


Figure 4. The effects of GR24 (strigolactone) on the stages of LRP development in *gpx2* (B) and *gpx3* (D) mutants compared to their respective Col-0 (A) and Col-3 (C) wild types. Black and white bars denote control and 2µM GR24 treatments respectively. Data are the mean ± SE of one biological replication. Different letters indicate significant differences $p<0.05$. Letter (a) denotes difference between treatments in the same genotype while letter (b) denotes difference between the mutant and wild-type after GR24 treatment.

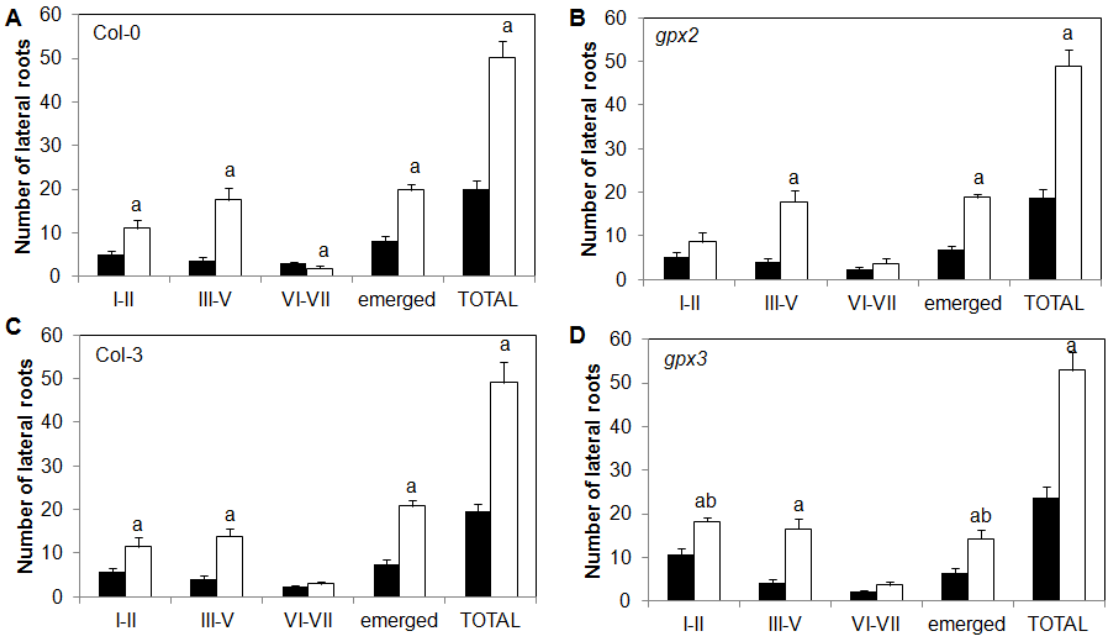


Figure 5. The effects of auxin on the stages of LRP development in the wild type controls (Figure 6A, C) and in the *gpx2* and *gpx3* mutants (Figure 6B, D). Black and white bars denote control and 1µM NAA conditions, respectively. Data are average ± SE of one biological replication. Different letters indicate significant differences $p<0.05$. Letter (a) denotes difference between treatments in the same genotype while letter (b) denotes difference between the mutant and wild-type after auxin treatment.

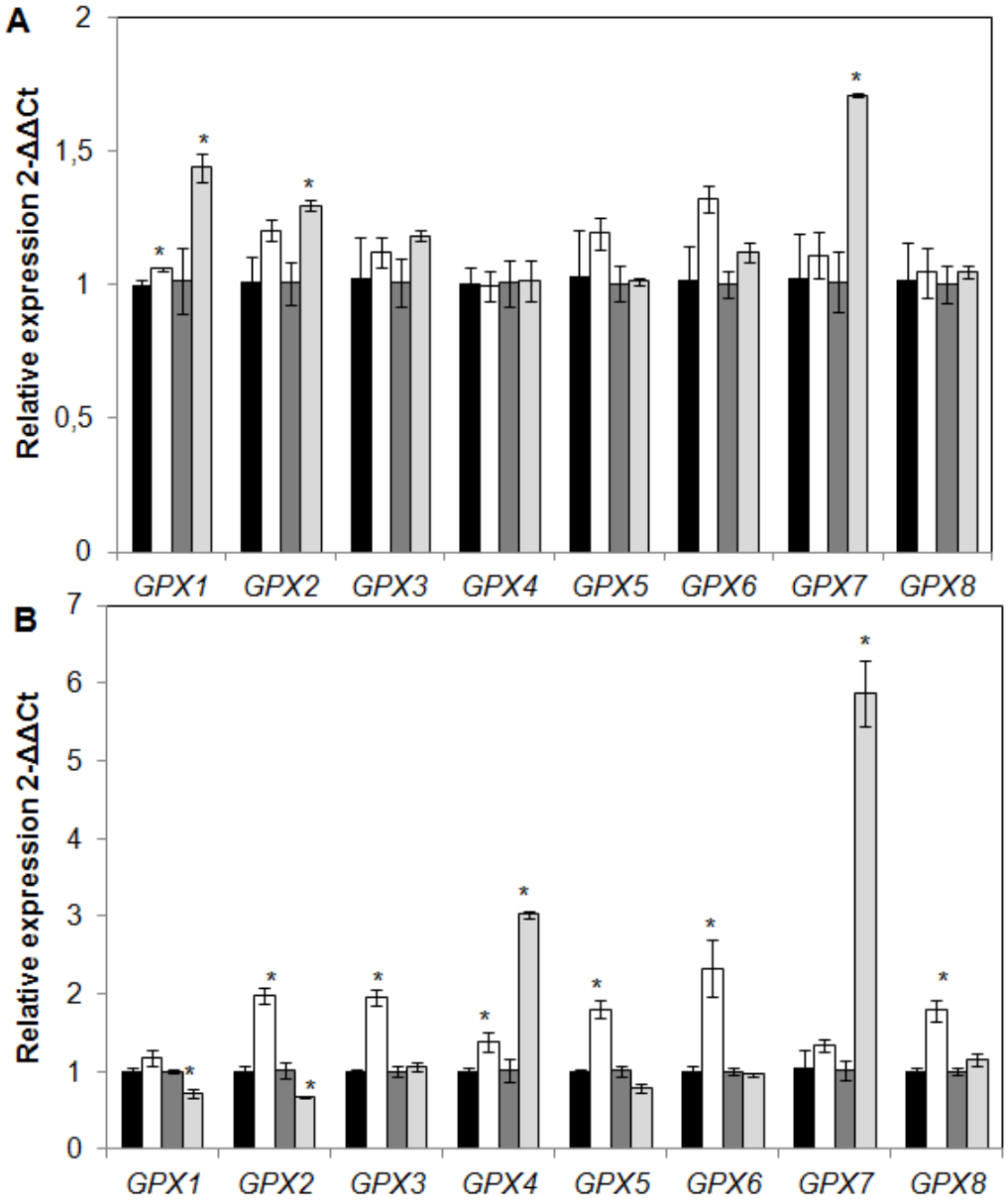


Figure 6. The effects of GR24 (2µM; A) and NAA (1µM; B) on the expression of *GPX* genes in Col-0 roots and shoots. Black and white bars denote shoots before and after hormone treatment, respectively. While dark and pale gray denotes roots after hormone treatment. Data are the mean ± SE of three biological replications, * indicates significant differences $p<0.05$ between.

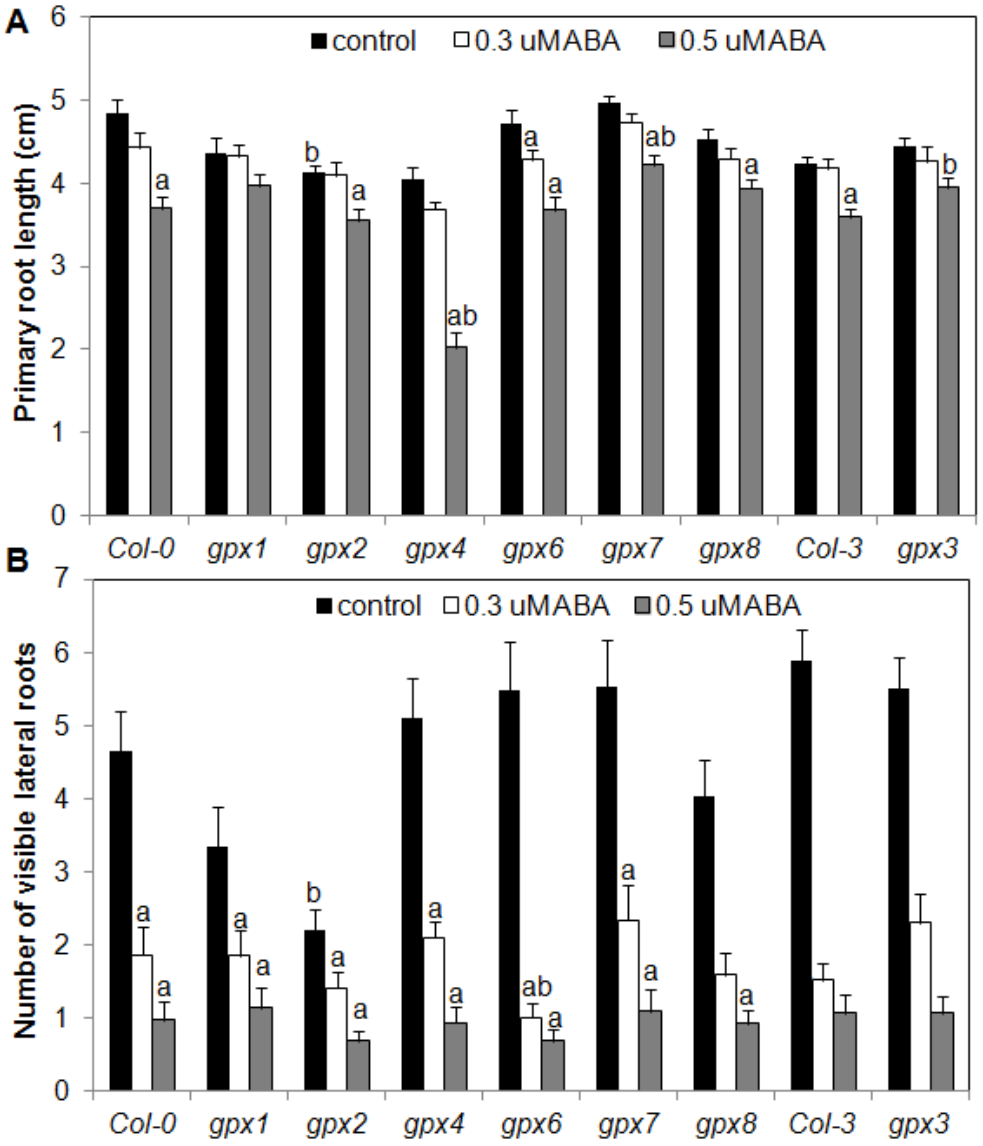


Figure 7. The effects of abscisic acid (ABA) on the growth of the primary root and the lateral roots in the different genotypes. The lengths of the primary roots (A) and number of lateral roots (B) were measured in the *gpx* mutant lines relative to the respective wild types (Col-0 and Col-3), together with the effect of ABA on these parameters. Data are average ± SE of one biological replication. Different letters indicate significant differences $p<0.05$. Letter (a) denotes difference between treatments in the same genotype while letter (b) denotes difference between the mutant and wild-type after treatment.

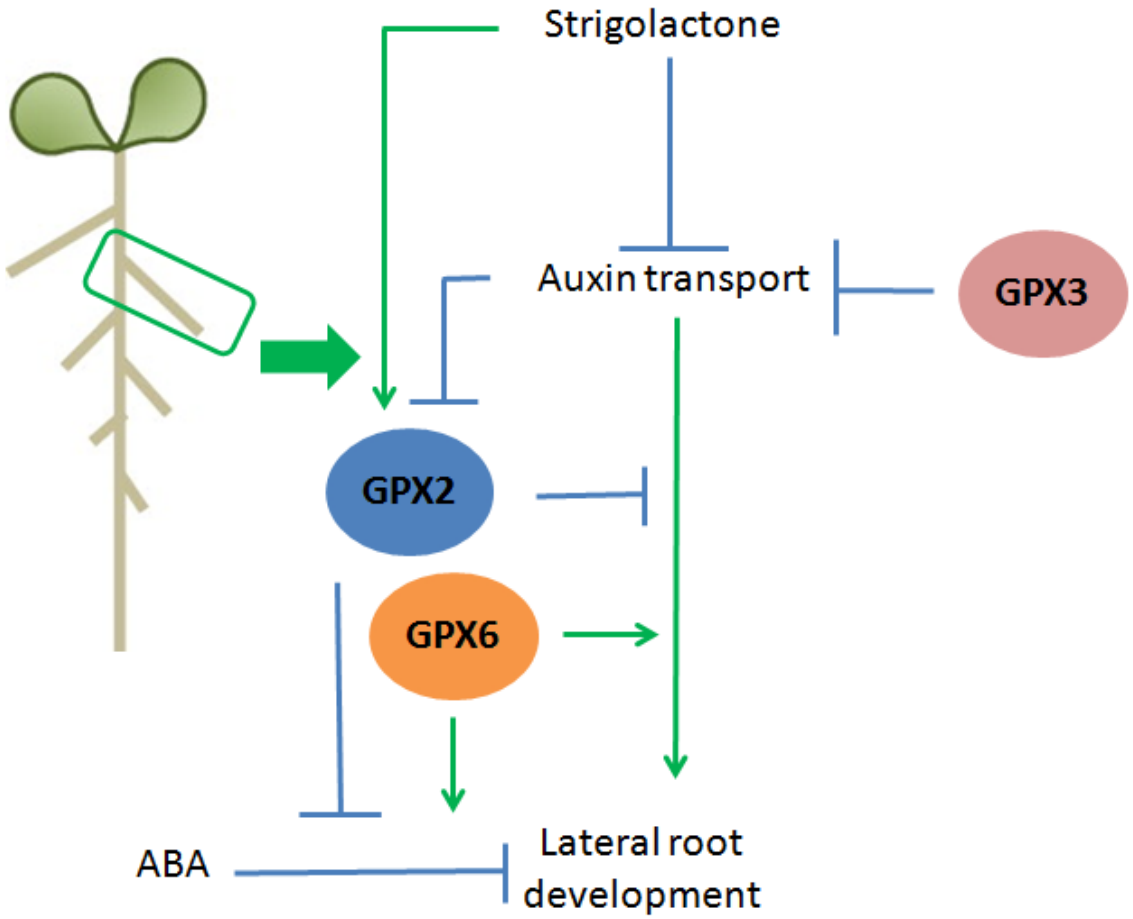
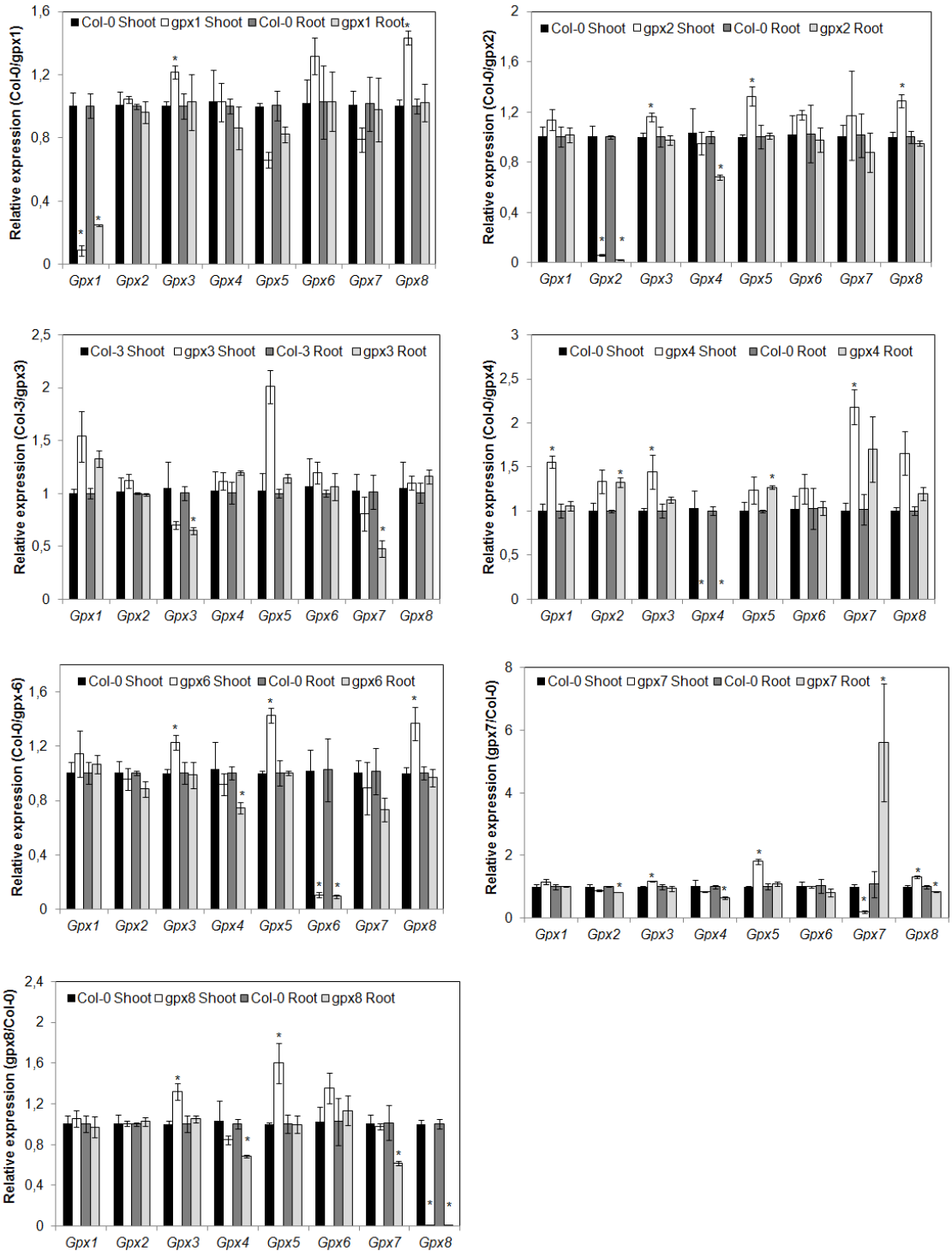
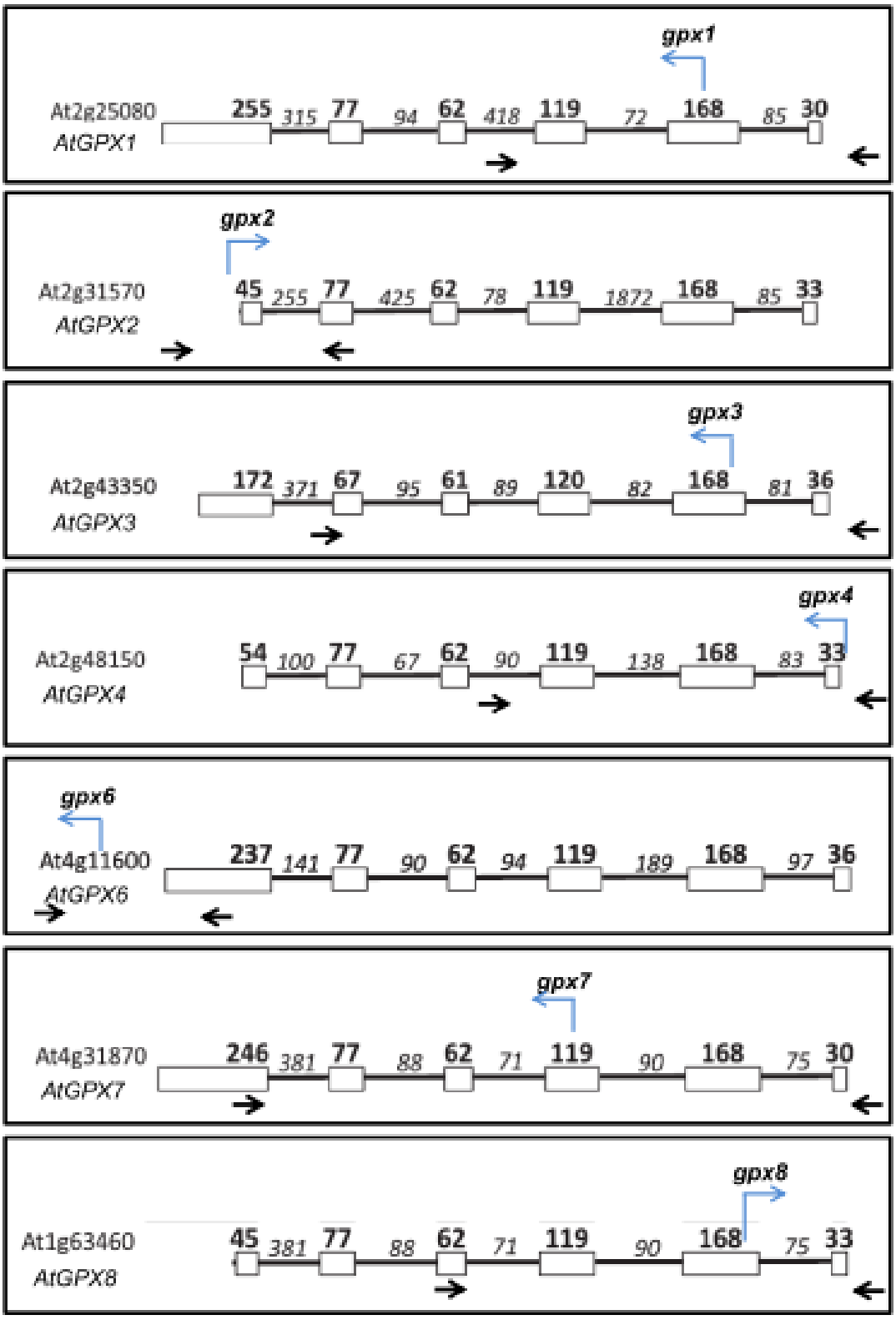


Figure 8. Schematic representation of the requirement for GPX2, GPX3 and GPX6 in hormone-dependent control of root architecture



Supplemental Figure 1. The relative expression of *GPX* genes in shoots: the black and white bars denote the wild type and mutants respectively, together with the relative expression of *GPX* genes in roots: dark and pale gray denote the wild type and mutants respectively). Data are the mean ± SE of three biological replications. * Asterisks indicate significant differences $p<0.05$.



Supplemental Figure 2. The insertions positions of T-DNA (blue arrows) in the *gpx* mutants and the primer positions used to detect the presence of the insert (black arrows).

Supplemental Table 1. Sequences of the primers used for the qPCR.

AGI Code	Name	Forward	Reverse
At2g25080	*GPX1*	5'-GTCTCCGGTAACCAAAAATG-3'	5'-GACGAGAAAGGTTGCTGAGG-3'
At2g31570	*GPX2*	5'-AAACTGCGTTGGGACAGG-3'	5'-CCCATGAAAAGACATCGAATAC-3'
At2g43350	*GPX3*	5'-GGGTCAATCAGCGAGCTAC-3'	5'-CGATGGCGAAGAAGGGTATC-3'
At2g48150	*GPX4*	5'-CCCACCTTTCTGGGTTCC-3'	5'-AACCATCGTGCCATAACG-3'
At3g63080	*GPX5*	5'-CAAAACGCTGCACCAGTC-3'	5'-GACCATCTTTGCCGACCAAG-3'
At4g11600*	*GPX6*	5'-GATGTTAACGGTGACAAAGCTG-3'	5'-TTGGTGCGAAACGATCG-3'
At4g31870	*GPX7*	5'-TCGGCCCATCATTGAGATTC-3'	5'-CTGCAGCCCTTGCATAGAC-3'
At1g63460	*GPX8*	5'-GAAAGGCAAATGGGGAATC-3'	5'-CAGCTTGACCGTTTTTGTC-3'
At3g18780	*ACTIN2*	5'CTGTACGGTAACATTGTGCTCAG-3'	5'-CCGATCCAGACACTGTACTTCC-3'

*(Queval et al., 2007).

CAPÍTULO 2

Caracterização da família GPX em arroz

Os resultados referentes à localização subcelular da família de GPX de arroz, juntamente com o padrão de expressão gênica dos seus cinco membros em plantas de arroz submetidos a diferentes estresses abióticos, tais como: frio, seca, luz UV-B e tratamento com H_2O_2 exógeno, além do perfil transcricional de seus membros durante o desenvolvimento vegetativo e reprodutivo serão apresentados nesse capítulo, adicionalmente à caracterização de plantas silenciadas especificamente para o gene *GPX3* (*GPX3s*). Além disso, o padrão de expressão de *Gus* controlado pela região promotora de GPX3 foi avaliado em plantas transgênicas.

Os resutados demonstram que os produtos dos genes de GPX de arroz estão localizados no citosol, mitocôndrias e cloroplastos, e são modulados positivamente na presença de H_2O_2 exógeno e frio. No entanto, são modulados negativamente em resposta a seca e a luz UV-B.

As plantas silenciadas para *OsGPX3* (plantas *GPX3s*) são menores do que plantas de arroz não transformadas, tanto na parte área quanto em raízes. Coerentemente, a atividade de sua região promotora foi detectada principalmente em tecidos de raiz, além de tricomas da panícula e embrião de sementes maduras.

Este estudo resultou em um artigo intitulado: "**The mitochondrial glutathione peroxidase (GPX3) is essential for H_20_2 homeostasis and for root and shoot development in Rice**", submetido ao periódico *Plant Science*. O artigo é apresentado a seguir.

The mitochondrial glutathione peroxidase GPX3 is essential for H_20_2 homeostasis and root and shoot development in rice

Gisele Passaia[a], Leila Spagnolo Fonini[a,b], Andréia Caverzan[a,b], Douglas Jardim Messeder[a], Ana Paula Christoff[a,b], Marcos Letaif Gaeta[c], Jorge Ernesto de Araujo Mariath[c], Márcia Margis-Pinheiro[a,b*]

[a] Department of Genetics, Federal University of Rio Grande do Sul, RS, Brazil.
[b] Biotechnology Center, Federal University of Rio Grande do Sul, RS, Brazil.
[c] Department of Botany, Federal University of Rio Grande do Sul, RS Brazil.

*Corresponding author. Address: Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, CEP: 915910-970, Porto Alegre - RS – Brazil, Telephone: +55 (51) 3308-9814. E-mail address: marcia.margis@ufrgs.br

Abstract

Glutathione (GSH) peroxidases (GPXs: EC 1.11.1.9 and EC1.11.1.12) are non-heme thiol peroxidases that catalyze the reduction of H_2O_2 or organic hydroperoxides to water, and they have been identified in almost all kingdoms of life. The rice glutathione peroxidase (*OsGPXs*) gene family is comprised of 5 members located in a range of sub cellular compartments. The *OsGPX* gene family is induced in response to exogenous H_2O_2 and cold stress. In contrast, they are down regulated in response to drought and UV-B light treatments. Transgenic rice plants have been generated that with decreased expression of the mitochondrial *OsGPX3*. These *GPX3s* plants showed shorter roots and shoots compared to non-transformed (NT) plants, and higher amounts of H_2O_2 mitochondrial release were observed in the roots of these plants cultivated under normal conditions. This accumulation of H_2O_2 is positively associated with shorter root length in *GPX3s* plants compared to NT ones. Moreover, *GPX3* promoter analysis indicated that it is mainly expressed in root tissue. These results suggest that silencing the mitochondrial *OsGPX3* gene impairs normal plant development and leads to a stress-induced morphogenic response via H_2O_2 accumulation.

Keywords: *Oryza sativa;* Poaceae; rice; GPX; glutathione peroxidase; oxidative stress.

1. Introduction

Whereas animals can move away from environmental adversities such as water deficit, high temperature or heavy metal pollution beyond others, plants are sessile organisms. As a result, the anti-oxidant and repair system of plants is considerably enhanced and diversified compared to bacterial or animal systems because plants also have to cope with a second source of reactive oxygen species (ROS) from chloroplasts, which is beyond the mitochondrial electron transport chain (Rouhier et al., 2008). The most abundant ROS produced during stress is hydrogen peroxide (H_2O_2). This compound is not only an oxidant but also a signal generated from superoxide ions through the action of superoxide dismutases (SOD) (Laloi et al., 2004). It is important that H_2O_2 levels are properly controlled under stress or normal conditions, and this is regulated by the orchestrated participation of a range of different enzymatic and non-enzymatic antioxidant systems. The non-enzymatic antioxidant compounds are represented by carotenoids, tocopherols, reduced glutathione (GSH) and ascorbate (AsA), while the enzymatic systems rely on superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), peroxiredoxins (PRXs) and glutathione peroxidases (GPXs) (Koh et al., 2007). Glutathione (GSH) Peroxidases (GPXs: EC 1.11.1.9 and EC1.11.1.12) are non-heme thiol peroxidases that catalyze the reduction of H_2O_2 or organic hydroperoxides to water, and they have been identified in almost all kingdoms of life (Herbette et al., 2007). In animals, GPXs are selenoproteins that contain selenocysteine at the catalytic site, whereas the plant enzymes do not bind selenium. The active site is composed of a catalytic triad formed by selenocysteine/cysteine, glutamine and tryptophan. This difference allows plants to preferentially reduce thioredoxin instead of GSH, which is opposite the preference of the enzymes in animals (Herbette et al., 2007; Toppo et al., 2008). The rice glutathione peroxidase (*OsGPXs*) gene family consists of 5 members. Phylogenic analysis has predicted that GPX is localized to the mitochondria (GPX1 and GPX3), chloroplasts (GPX4) and cytosol/endoplasmic reticulum (GPX2 and GPX5) (Margis et al., 2008b).

There is increasing evidence that GPXs are able to interact with other proteins, conferring peroxide-induced oxidation in addition to their well-established ROS scavenging function. For example, in yeast, GPX3 activates the Yeast Activation Protein 1 (Yap-1) transcription factor via such an oxidation-induced event to promote the activation of genes encoding thiol-reducing and antioxidant proteins (Delaunay et al., 2002). In

Arabidopsis thaliana, GPX3 links ABA and H_2O_2 signaling during stomatal closure by interacting with protein phosphatase type 2C (PP2C) proteins, such as Abscisic acid Insenstive (ABI) 1 and ABI2 (Miao et al., 2006c).

Rice roots are typical of semi-aquatic plants in that they have specialized tissues that allow for root growth during flooding conditions (Rebouillat et al., 2008). The aerenchyma is a tissue that is specially designed to promote gas exchange from the shoot, and it also acts as a reservoir for oxygen, which is required for root respiration under flooding conditions. Characteristics such as deep, thick, branched root systems are correlated with better survival and crop productivity. A range of phytohormones, such as abscisic acid (ABA) and auxin, are known to be involved in root development and architecture. The accumulation of ABA in roots under water stress is required for maintaining primary root elongation (Sengupta et al., 2011), and auxin transport promotes root growth via auxin maxima conditions (Grieneisen et al., 2007; Robert and Friml, 2009). Moreover, it was recently shown that ABA accumulation modulates auxin transport in the rice and Arabidopsis root tip to maintain primary root elongation under water stress (Xu et al., 2012). ABA also triggers H_2O_2 production via plasma membrane NADPH oxidases (RbohD and RbohF), which increase cytosolic Ca^{2+}, leading to stomata closure (Kwak et al., 2006). On the other hand, auxin promotes ROS production through NADPH oxidase activation, which plays a role in root gravitropism; specifically, H_2O_2 causes root curvature as a downstream component of the auxin-mediated signaling pathway (Joo et al., 2001). To date, the functions of rice GPXs have not been reported. The present study was undertaken to assess the functional characterization of the *GPX* gene family in rice, especially GPX3, using a knockdown gene silencing approach to abolish *GPX3*. These transgenic plants displayed short shoot and root phenotypes and increased H_2O_2 production compared to NT plants, indicating that GPX3 is involved in rice development processes in an H_2O_2-dependent manner.

2. Material and Methods

2.1. Vectors construction and plant transformation

The target gene sequence from *OsGPX3* (LOC_Os02g44500; 197bp) was inserted into the pANDA RNAi vector (Miki and Shimamoto, 2004) using the gateway LR reaction (Invitrogen®). cDNAs were used as a template for PCR reactions. The following primer pairs were used to amplify the sequence: RNAiGPX3: 5'-CACCACCGCCCCGTTCATCTCT-3' and 5'- ATGCTGAACAGCGCGAAC-3'. The hairpin RNA produced by the pANDA vector is driven by the maize ubiquitin promoter. To verify the GPX3 promoter expression pattern, a 2 Kb fragment upstream of the start codon was amplified by PCR, cloned into pENTR (Invitrogen®) and recombined with pHGWFS7 (Karimi et al., 2005), producing the vector proGPX3. The proGPX3 vector contains the *Gus* and *gfp* reporter genes and also *hpt* as selection marker gene that confers plant resistance to hygromycin. The following primers were used to amplify the *hpt* gene: 5´-CACCCGTTTCTCGATGACATGACG-3' and 5´-ATAGCGGTAAGTCCGGTGTG-3'. The plasmids were introduced into rice calli via *Agrobacterium tumefaciens*-mediated transformation, as described by Upadhyaya, 2002.

Using cDNA from leaves, *OsGPX* genes (1 to 5: LOC_Os04g46960, LOC_Os03g24380, LOC_Os02g44500, LOC_Os06g08670, LOC_Os11g18170) were amplified using the following primers: *GPX1*: 5'- ATGGCCGCCGCGCCGTCC-3' and 5'-AGAGCTCCCAAGCAGCTTCTTGATA-3'; *GPX2*: 5'- ATGGGGGCGGCGGAATCC-3' and 5'- ATCCTCGAGCGCCTTCAGGATGTC-3'; *GPX3*: 5'-ATGGCCACCGCCGCCTCC-3' and 5'- AGAACTTCCAAGCAGCTTCTTGATG-3'; *GPX4*: 5'- ATGGCGTCCACCACCACC-3' and 5'- GGGCTGCCTCACCATCAGTA-3'; *GPX5*: 5'- ATGGCGGCTACAACTACAAG-3' and 5'-CGAGGTCCCAAGTAGCTTCT-3'. PCR products were cloned into the pART7-HA-YFP plasmid (Galván-Ampudia and Offringa, 2007) using Gateway technology (Invitrogen®), in fusion with the Yellow Fluorescent Protein (YFP) coding sequence at its N-terminus, under control of 35S promoter of CaMV. The constructs were used to transform rice protoplasts.

Protoplast isolation was performed as described by Chen et al., (2006), with minor modifications, and protoplast transformation was performed as described by Tao et al. 2002 (Tao et al., 2002). After transformation, the protoplasts were incubated for 24-48

hours in the dark at 28 °C before imaging. To evaluate fluorescence, an Olympus FluoView 1000 confocal laser-scanning microscope, equipped with a set of filters capable of distinguishing between green and yellow fluorescent protein (EGFP and EYFP, respectively) and plastid autofluorescence, was used. The images were captured with a high-sensitivity photomultipler tube detector.

2.2. Plant growth, abiotic stress and hormone treatments

Rice plants (*Oryza sativa* L. ssp. Japonica cv. Nipponbare) were used in this study. The plants were grown in a growth chamber with supplemental lighting (8 hours dark/16 hours light; 150 mol m^{-2} s^{-1}) at 28 ºC or in a greenhouse (sunlight), submerged in H_2O. Seeds were germinated in H_2O at 28 ºC in the dark. Four-day-old seedlings were transferred to plastic pots containing Furlani's solution (48 mg/L N–NO_3^-; 12 mg/L N–NH_4^-; 200 mg/L Ca; 200 mg/L K; 40.6 mg/L Mg; 8.0 mg/L P; 151 mg/L S; 234 mg/L Cl; 4.85 mg/L Fe; 0.67 mg/L Mn; 0.36 mg/L B; 0.20 mg/L Zn; 0.05 mg/L Cu; and 0.11 mg/L Mo) and grown at 28 ± 2 ºC, with a 12 hours photoperiod.

Non-transgenic rice plants were used to evaluate *OsGPX* gene expression during vegetative and reproductive development. Rice seeds were sterilized and germinated on filter paper, and after emergence, they were transferred to vermiculite. Leaves and root tissues were sampled 7, 14 and 30 days after germination (DAG). Moreover, panicle samples were taken during reproductive development (R), based on the following stages (Counce et al., 2000): R2, R3, R4, R5, R6, R7, and R8. *OsGPX* gene expression was evaluated in response to exogenous H_2O_2 treatment, cold, UV-B light and drought stress.

To analyze the response of rice plants to exogenous H_2O_2 treatment, plants were germinated and grown in 1/2 Murashige and Skoog (MS) medium. After 4 weeks of growth, the seedlings were transferred to liquid MS supplemented with 10 µM H_2O_2 and incubated for 2, 4, and 8 hours. Control plants were maintained in MS without H_2O_2.

For cold stress, 3-week-old rice plants were exposed to a 10 ºC climate for 24 hours. Control plants remained in the conditions described previously. Shoot samples were taken after this period.

For aluminum treatment, 4-day-old seedlings were grown in nutrient solution supplemented with 20 ppm $AlCl_3$, and plant material was sampled at 4 hours and 8 hours after treatment.

For UV-B light treatment, 3-week-old rice plants were exposed to UV-B irradiation (254 nm) for 4 hours. Samples were collected immediately after exposure (time 0) and at 6 hours after exposure.

For drought stress, seedlings were grown to the four-leaf stage on soil supplemented with water. After this period, the seedlings were separated into two lots: control and drought stressed. The control lot continued to receive a normal supply of water, whereas water was withheld from the stressed seedlings for 6 and 15 days. After this period, plant material was collected from both lots of plants.

Transgenic rice plants in which *OsGPX3* was silenced were obtained via *Agrobacterium tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp. Japonica cv. Nipponbare), and they were induced from seeds and cultivated in NB medium at 28 ºC in the dark (Hiei et al., 1994). T1 rice seeds from *OsGPX3s* plants and non-transformed (NT) were germinated in MS medium (Sigma-Aldrich) supplemented with hygromycin under controlled conditions (150 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 25 °C, 80% relative humidity and a 12 hours photoperiod). Non-transformed rice and *GPx3s* seeds were grown in MS media with and without hygromycin (30 mg/ml), respectively. After 8 days, seedlings were transferred to Furlani's solution and grown at 28 ± 2 °C with a 12 hours photoperiod for 30 days. The pH of the solution was adjusted to 5.4 using 0.1 N HCl. After 10 days, the shoot and root lengths were measured. Three biological replicates were performed for each experiment; a pool of at least three plants per replicate was used.

2.3. Analysis of the Gpx3 promoter expression pattern in rice plants

Transgenic plants were used to assess the expression of the Gus gene under the control of the GPX3 promoter (promGPX3-GUS). Leaves, roots, stem and panicles were sampled and analyzed using the *X-Gluc* histochemical assay (Fermentas®), as previously described (Jefferson et al., 1987), with minor modifications. After Gus staining, the samples were clarified with graded ethanol series (30% to 70%) and analyzed in stereomicroscope and bright field microscopy.

2.4. Determination of mitochondrial H_2O_2 Release

The amount of H_2O_2 released by rice roots was determined by the Amplex Red oxidation method, as described previously (Smith et al., 2004). Briefly, roots (20 mg) were incubated in 20 mM MES buffer (pH 6.0) supplemented with 10 mM Amplex Red and 5 units/mL horseradish peroxidase. Fluorescence was monitored at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H_2O_2.

2.5. Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated using the TRIZOL reagent (Invitrogen®), according to the manufacturer's protocol, and the quality of the resulting RNA was assessed by gel electrophoresis. To eliminate contaminant genomic DNA, samples were treated with RQ1 RNase-free DNase, according to the manufacturer's instructions (Promega, Madison, WI, USA). Synthesis of first-strand cDNA was performed by incubating 1 µg of total RNA with the M-MLV Reverse Transcriptase (Promega) and a 24-polyTV primer (Invitrogen®). After cDNA synthesis, the samples were diluted 100-fold in sterile water. Primers were designed to produce DNA fragments ranging from 180-250 bp. PCR amplifications were performed using specific primers for *OsGPX1*: 5'- AGCAACCTGCACTTATGCACT-3' and 5'- CAGCAAGGAAATTTATTGACATGA-3'; *OsGPX2*: 5'- CTGGTTGGGTAGGCACTGTT-3' and 5'- TGCAAACACAAACCTTACGCTAC-3'; *OsGPX3*: 5'- TTGCATTGAGCACTTGGAAC-3' and 5'- AGGGGCAAAGTGATGCAGTA-3'; *OsGPX4*: 5'- CTGTACATATGCCTTGCCTCA-3' and 5'-GTTACAGGGGCCAGATAAGC-3'; *OsGPX5*: 5'- AAGATTGAGAATGATATCCAGAAGC-3' and 5'- GCAAACCACATTCTTACGAACA-3'. The following primers were used as internal controls to normalize the amount of mRNA present in each sample: *OsActina2*: 5'- GGACGTACAACTGGTATCGTGTT-3' and 5'-GTTCAGCAGTGGTAGTGAAGGAG-3'; *OsFdh3*: 5'-TTCCAATGCATTCAAAGCTG-3' and 5'- CAAAATCAGCTGGTGCTTCTC-3'; *OseF1α*: 5'- TTTCACTCTTGGTGTGAAGCAGAT-3' and 5'- GACTTCCTTCACGATTTCATCGTAA-3'. qPCR assays were carried out using the Step One plus PCR System (Applied Biosystems, Foster City, CA, USA). Three biological replicates were performed for each experiment; a pool of at least three plants per replicate was used. Also, a total of 4 technical replicates were performed for each reaction, and expression data analyses were performed after comparative quantification of amplified

products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

2.6. Statistical analysis

The data represent the means +/- standard error of the mean of the three housekeeping genes (SEM). Statistical analysis was performed using Student's Test, and a p value of 0.05 was considered to be statistically significant.

3. Results

3.1. GPX Proteins are mainly localized in plant cell organelles

Margis *et al*. (2008) identified 6 GPX genes in the rice genome. Subsequent analysis revealed that OsGPX6 is actually one of the four splice forms of OsGPX1. In the referenced work, *in silico* analysis predicted that OsGPX1 and OsGPX3 were localized in the mitochondria, OsGPX2 in cytosol, OsGPX4 in chloroplasts and OsGPX5 in cytosol anchored at the endoplasmic reticulum. In the present work, we experimentally analyzed the sub-cellular localization of the rice GPX family. We constructed a translational fusion of different OsGPX that expressed YFP, driven by the CaMV 35S promoter, in rice protoplasts. Confocal analysis of protoplasts expressing 35S-OsGPX1::YFP, 35S-OsGPX2::YFP and 35S-OsGPX3::YFP, 35S-OsGPX4::YFP and 35S-OsGPX5::YFP fusions revealed that YFP fluorescence was localized at the mitochondria (Fig. 1A), cytosol and chloroplasts (Fig. 1B), mitochondria (Fig. 1C), chloroplasts (Fig. 1D) and cytosol/endoplasmic reticulum and chloroplasts (Fig. 1E), respectively. Tetramethyl rhodamine ethyl ester perchlorate (TMRE), a mitochondria-specific fluorescent marker, was used in rice protoplasts transformed with 35S-OsGPX1::YFP and 35S-OsGPX3::YFP constructs to confirm their mitochondrial localization. 35S-OsGPX2::YFP and 35S-OsGPX5::YFP were also present beyond the cytosol or cytosol/endoplasmic reticulum, respectively, in chloroplasts.

3.2. GPX gene expression is mainly regulated by exogenous H_2O_2

To verify how exogenous H_2O_2 modulates rice *GPXs* genes, rice plants were submitted to 10 µM H_2O_2 treatment, and RT-qPCR analysis was performed. All of the *GPX* genes examined were significantly induced after 2, 4 and 8 hours of treatment, except Gpx4, which was induced after 8 hours of treatment (Fig. 2A). Moreover, to elucidate how the *GPX* gene family responds to abiotic stress, the expression of *GPX* genes was analyzed after exposure to cold, drought, UV-B light, and aluminum. Rice *GPX*s were not modulated by 20 ppm aluminum after 4 or 8 hours in roots (data not shown). On the other hand, *GPX1*, *GPX3* and *GPX5* expression were significantly induced when plants were subjected to a 10 ºC climate for 24 hours (Fig. 2B). Rice plants cultivated without water for 15 days displayed a significant reduction in the expression of *GPX4* and *GPX5* (Fig. 2C). Regarding UV-B light treatment, the expression of *GPX3* and *GPX4* was significantly

reduced immediately after UV-B light exposure and after 6 hours, while the expression of *GPX1*, *GPX2* and *GPX5* was significantly reduced only after 6 hours of UV-B light exposure (Fig. 2D).

GPX4 showed the highest expression in the leaves 14 and 30 DAG (Days after Germination) followed by *GPX3* at 14 DAG. In roots, the most highly expressed gene was *GPX3* (Suppl. Fig. 1A). During reproductive development, all of the *GPX* genes showed very low expression levels at the R1 and R2 stages, while *GPX5* presented the highest level of expression at R3, R4 and R5. Moreover, the expression of *GPX2* and *GPX4* was higher at stages R4 and R5, respectively (Suppl. Fig. 1B).

3.3. OsGPX3 is mainly expressed in the root tissues of rice plants

Rice calli, transformed with the proGPX3-Gus construct, were cultured with hygromycin. Gus positive calli were selected, and the plants were regenerated and incubated in *X-gluc* to visualize *GPX3* promoter expression patterns. Tissue from the leaves, roots, stems and spikelets of two different lineages were evaluated. The proGPX3 expression showed Gus staining in the phloem region of adventitious root of stem primordial basis (Fig. 3A, B) and apices of young roots, specifically in the differentiation zone (Fig. 3C, D). Moreover, Gus staining was visualized in three cellular trichomes in palea/lemma of the spikelet (Fig. 3 E, F) and in the embryo of mature seeds (Fig. 3G).

3.4. Post-transcriptional knockdown of OsGPX3 affects root and shoot growth

To determine the functional role of mitochondrial (*OsGPX3*) glutathione peroxidase in rice, the *OsGPX3* gene was specifically silenced using inverse repeat (IR) constructs transcribing dsRNA (hairpin). In rice, the nucleotide sequence of *GPX* genes is well conserved. Identities among the family members vary from 46% to 81%, and genes encoding proteins of the same sub-cellular compartment share long, identical, contiguous sequence stretches (up to 30 bp in length). Because of the high similarity in the coding sequence region between *GPX* genes, we designed an IR construct transcribing dsRNA for a specific 197 bp region of the *OsGPX3* 5'UTR (Table 1). Rice calli were transformed with the RNAi*OsGPX3* construct, generating 6 transgenic lines. *GPX3*s plants showed lower height compared to NT (Fig. 4A). The mRNA level of *GPX3*s was reduced to 5% of the control NT plants (Fig. 4B). Evaluation of the other family members revealed a 40%

reduction in *OsGPX2* expression in the *GPX3*s transgenic plants compared to NT plants, but none of the other genes were altered significantly (Fig. 4C). We germinated three *GPX3s* line and non-transformed plants in MS media, with and without hygromycin, respectively. After 8 days, the seedlings were transferred to a nutrient solution for 10 days, and the shoot and root lengths were measured. All of the *GPX3s* plant lines showed shorter shoots (Fig. 4D) and roots (Fig. 4E) compared to control NT plants.

3.5. Silencing of GPX3 expression leads to higher levels of mitochondrial H_2O_2 release

Three lines of *GPX3s* and non-transformed rice plants were germinated in MS media for 8 days and then cultivated for 10 days in nutrient solution. Then, H_2O_2 mitochondrial release was measured. *GPX3s* plants released an average of 20 (lines 6 and 8) and 36 (line 7) times more H_2O_2 in their roots compared to wild type plants (Fig. 5.A). While NT plants produced 0.00627 pmol H_2O_2 mg tissue^{-1} min^{-1}, the *GPX3s-6* and *GPX3-8* transgenic lines produced 0.1302 and 0.1208 pmol H_2O_2 mg tissue^{-1} min^{-1}, respectively, and *GPX3s-7* produced 0.2272 pmol H_2O_2 mg tissue^{-1} min^{-1}. Moreover, a positive ratio existed between ROS production and root length (Fig. 5B), where the highest production of H_2O_2 correlated with short roots in all transgenic lines. However, the wild type plants showed longer root length and less H_2O_2 production. Notably, *GPX3s-7* released the highest amount of ROS, and it also presented the shortest root length in average between the lines studied (Fig. 5B).

4. Discussion

Using *in silico* analysis, we previously predicted the sub-cellular localization for all members of the OsGPX family (Margis et al., 2008b). In the present study, our data confirmed those predictions, and found that OsGPX2 and OsGPX5 were located in the chloroplasts of rice protoplasts, in addition to their expected cytosolic localization (Fig. 1B, E). This unexpected localization was also reported for ascorbate peroxidase, glutathione reductase and monodehidroascorbato reductase in Arabidopsis, that demonstrated dual targeting to mitochondria and plastids (Chew et al., 2003). Additional studies are necessary to confirm the chloroplast localization of OsGPX2 and 5.

Expression analysis showed the strong induction of all *OsGPX* genes in response to H_2O_2 treatment, suggesting that rice GPXs participate in maintaining ROS homeostasis and redox signaling (Fig. 2A). In contrast, we did not observe any change in GPX expression in rice roots treated with Al (data not shown). These results are not in agreement with what has been reported for rye roots (Milla et al., 2002), where *GPX* transcripts were rapidly induced by Al after 4 hours and peaked at 8 hours, before decreasing to normal levels. *OsGPX* was down regulated after drought treatment, in contrast with what has been observed for *OsAPX* genes under the same conditions; among the 8 *OsAPX* members, 5 were induced after 15 days of drought (Rosa et al., 2010). The absence of modulation or repression of *OsGPX* genes under abiotic stress could be explained by the strength of the cell redox state changes during different abiotic stresses. In barley, GPX was not affected by cold, heat or drought treatment, and only a slight increase was observed in roots treated with salt and H_2O_2 (Halusková et al, 2009). In animals, GPXs function as key enzymes in the detoxification of ROS, especially H_2O_2. In plants, detoxification of ROS is mainly attributed to the catalases and enzymes of the ascorbate-glutathione cycle. Moreover, it has been reported that during severe and persistent stress, ascorbate peroxidases are inhibited, and GPXs become the main H_2O_2 scavenging enzymes (Halusková et al, 2009). Similarly, in salt-sensitive citrus cells, ascorbate peroxidases decreased, whereas GPX activity increased with time (Gueta-Dahan et al., 1997). Previously, our group demonstrated that *OsGPX* expression was induced in cytosolic ascorbate peroxidase knockdown rice plants under heat stress and normal conditions (Bonifacio et al., 2011). Furthermore, GST and GPX detoxify other toxic organic hydroperoxides, which increase during severe and permanent stress-induced lipid peroxidation. In the case of strong stress conditions, where

basal antioxidant mechanisms are exhausted, more effective responses are activated by GST/ GPX or GPX (Halusková et al, 2009). In rice, the 5 *GPX* members appear to play different roles during plant development and in response to abiotic stress.

The evaluation of *OsGPX* gene expression during rice vegetative development revealed that *OsGPX3* is the predominant isoform expressed in roots, while *OsGPX4* showed the highest expression in leaves, followed by *OsGPX3* (Suppl. Fig. 1A). The expression of the *OsGPX3* promoter was detected in the adventitious root of stem primordia basis (Fig. 3A). More specifically, the *OsGPX3* promoter was found in the phloem region of the provascular meristem (Fig. 3B) and was not detected in the stem primordia itself. The promoter activity was also detected in young adventitious roots (Fig. 3C), specifically in the elongation zone (Fig. 3D). This observation is also agrees with the RiceXPro database analysis for this gene, which indicated that *OsGPX3* is expressed mainly in the elongation zone and maturation zone I (Suppl. Fig. 2D), specifically the endodermis, pericycle and stele (Suppl. Fig. 2E). The activity of GPX-related genes, in response to stress, has been found in trichomes of *Nicotiana tabacum*, indicating the role of these structures in the elimination of ROS (Harada et al., 2010). Interestingly, strong Gus staining was also detected in the embryo of mature rice seed, which could be important for radicle and coleoptile emergence (Counce et al., 2000). In all cases which expression was detected in OsGPX3-GUS represent tissues undergoing intense cell division and differentiation. This observation is in agreement with Herbette *et al.* (Herbette et al., 2004), which suggest that GPX can protect dividing cells from oxidative stress, helping to maintain the mitotic activity.

To understand the role of mitochondrial *OsGPX3* in rice antioxidant metabolism, this gene was silenced in transgenic lines. The reduction of *OsGPX3* expression by approximately 97% did not change the response of other *OsGPX* members, except *OsGPX2*, which was down regulated (Fig. 4C). *OsGPX3* knockdown effectively reduced the shoot and root lengths of 18-day-old T1 rice plants compared to wild type plants (Fig. 4D, E, respectively). Moreover, the flux of H_2O_2 release from rice roots was approximately 20 times more intense compared to NT plants (Fig. 5A). Interestingly, the highest H_2O_2 production also correlated with reduced root length (Fig. 5B). Thus, H_2O_2 fine-tuning seems to be crucial for the determination of cell proliferation/elongation or cell arresting. For example, auxin induces H_2O_2 in the root tip and the apical half of the root elongation zone, driving root gravitropism in maize (Joo et al., 2001). *OsGPX3* promoter analysis showed Gus staining in the apices and elongation zones of young roots, which suggests

that *OsGPX3* knockdown leads to increased production of H_2O_2, impairing root growth. Moreover, it was recently suggested that root growth inhibition was a consequence of redox homeostasis disturbance and is a general stress-induced morphogenic response (SIMR) of roots to different stresses (Potters et al., 2009). As *OsGPX3* knockdown plants produce a high level of H_2O_2, this theory could explain their shorter root and shoot phenotype. Key elements in the ontogenesis of the SIMR phenotype appear to be stress-affected gradients of ROS, antioxidants, auxin and ethylene. These gradients are integrated on the cellular level, affecting cell division, cell elongation and/or cell differentiation (Potters et al., 2009). Additionally, a moderate increase in ROS production caused root growth inhibition and the induction of GPX activity in cells through cell wall crosslinking (Córdoba-Pedregosa et al., 2007). Taken together, our results suggest that mitochondrial OsGPX3 is important for normal rice development and that the shorter root and shoot phenotype of *GPX3*s plants could be explained by the increased H_2O_2 levels and conversion to a stress-induced morphogenic response. The functional analysis of genes belonging to the *GPX* gene family in rice, which are an important monocot species, could aid our understanding of the stress response processes and other unique characteristics of this group.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the International Centre for Genetic Engineering and Biotechnology (ICGEB). We are grateful to Universidade Federal do Ceará (UFC), Professor Joaquim Albenísio da Silveira, Fabrício E.L. Carvalho and Yugo Lima Melo for rice seed propagation and Carolina Werner Ribeiro for useful discussions.

References

Bonifacio A, Martins MO, Ribeiro CW, Fontenele A V, Carvalho FEL, Margis-Pinheiro M, Silveira J a G (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant, cell & environment **34**: 1705–22

Chew O, Whelan J, Millar a H (2003) Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. The Journal of biological chemistry **278**: 46869–77

Chen S., Tao L., Zeng L., Vega-Sanchez M.E, Kenji Umemura WG-L (2006) A highly efficient transient protoplast system for analyzing defence gene expression and protein – protein interactions in rice. Molecular Plant Pathology **7**: 417–427

Córdoba-Pedregosa MC, Villalba JM, Córdoba F, González-Reyes J a. (2007) Changes in Growth Pattern, Enzymatic Activities Related to Ascorbate Metabolism, and Hydrogen Peroxide in Onion Roots Growing Under Experimentally Increased Ascorbate Content. Journal of Plant Growth Regulation **26**: 341–350

Counce P a., Keisling TC, Mitchell AJ (2000) A Uniform, Objective, and Adaptive System for Expressing Rice Development. Crop Science **40**: 436

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111**: 471–81

Galván-Ampudia CS, Offringa R (2007) Plant evolution: AGC kinases tell the auxin tale. Trends in plant science **12**: 541–7

Grieneisen V a, Xu J, Marée AFM, Hogeweg P, Scheres B (2007) Auxin transport is sufficient to generate a maximum and gradient guiding root growth. Nature **449**: 1008–13

Gueta-Dahan Y, Yaniv Z, Zilinskas B a, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. Planta **203**: 460–9

Harada E, Kim J-A, Meyer AJ, Hell R, Clemens S, Choi Y-E (2010) Expression profiling of tobacco leaf trichomes identifies genes for biotic and abiotic stresses. Plant & cell physiology **51**: 1627–37

Herbette S, Brunel N, Prensier G, Julien J-L, Drevet JR, Roeckel-Drevet P (2004) Immunolocalization of a plant glutathione peroxidase-like protein. Planta **219**: 784–9

Herbette S, Roeckel-Drevet P, Drevet JR (2007) Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. The FEBS journal **274**: 2163–80

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant journal : for cell and molecular biology **6**: 271–82

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: ,B-glucuronidase. **6**: 3901–3907

Joo JH, Bae YS, Lee JS (2001) Role of Auxin-Induced Reactive Oxygen Species in. Plant Physiology **126**: 1055–1060

Karimi M, De Meyer B, Hilson P (2005) Modular cloning in plant cells. Trends in plant science **10**: 103–5

Koh CS, Didierjean C, Navrot N, Panjikar S, Mulliert G, Rouhier N, Jacquot J-P, Aubry A, Shawkataly O, Corbier C (2007) Crystal structures of a poplar thioredoxin peroxidase that exhibits the structure of glutathione peroxidases: insights into redox-driven conformational changes. Journal of molecular biology **370**: 512–29

Kwak JM, Nguyen V, Schroeder JI (2006) The Role of Reactive Oxygen Species in Hormonal Responses 1. **141**: 323–329

Laloi C, Apel K, Danon A (2004) Reactive oxygen signalling: the latest news. Current opinion in plant biology **7**: 323–8

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) **25**: 402–8

Margis R, Dunand C, Teixeira FK, Margis-Pinheiro M (2008) Glutathione peroxidase family - an evolutionary overview. The FEBS journal **275**: 3959–70

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. The Plant cell **18**: 2749–66

Miki D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant & cell physiology **45**: 490–5

Milla MAR, Butler E, Huete AR, Wilson CF, Anderson O, Gustafson JP (2002) Expressed Sequence Tag-Based Gene Expression Analysis under Aluminum Stress in Rye 1 [w]. **130**: 1706–1716

Potters G, Pasternak TP, Guisez Y, Jansen M a K (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant, cell & environment **32**: 158–69

Rebouillat J, Dievart a., Verdeil JL, Escoute J, Giese G, Breitler JC, Gantet P, Espeout S, Guiderdoni E, Périn C (2008) Molecular Genetics of Rice Root Development. Rice **2**: 15–34

Robert HS, Friml J (2009) Auxin and other signals on the move in plants. Nature chemical biology **5**: 325–32

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira J a G, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry **71**: 548–58

Rouhier N, Koh CS, Gelhaye E, Corbier C, Favier F, Didierjean C, Jacquot J-P (2008) Redox based anti-oxidant systems in plants: biochemical and structural analyses. Biochimica et biophysica acta **1780**: 1249–60

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nature Protocols **3**: 1101–1108

Sengupta D, Kannan M, Reddy AR (2011) A root proteomics-based insight reveals dynamic regulation of root proteins under progressive drought stress and recovery in Vigna radiata (L.) Wilczek. Planta **233**: 1111–27

Smith AMO, Ratcliffe RG, Sweetlove LJ (2004) Activation and function of mitochondrial uncoupling protein in plants. The Journal of biological chemistry **279**: 51944–52

Tao L, Cheung AY, Wu H (2002) Plant Rac-Like GTPases Are Activated by Auxin and Mediate Auxin-Responsive Gene Expression. The Plant Cell **14**: 2745–2760

Toppo S, Vanin S, Bosello V, Tosatto SCE (2008) Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. Antioxidants & redox signaling **10**: 1501–14

Upadhyaya NM, Zhou X-R, Zhu Q-H, Eamens A, Wang M-B, Waterhouse PM, Dennis ES (2002) Transgenic Rice. In Transgenic Cereals, ed O'Brien L , Henry RJ, AACC, Minnesota: 28-87.

Xu W, Jia L, Shi W, Liang J, Zhou F, Li Q, Zhang J (2012) Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. The New phytologist.

Table 1. Similarities among the sequences of the hairpin construction and the rice *GPX* genes. The number of contiguous nucleotides shared between each rice *GPX* gene and the target sequence of the hairpin construct are indicated in parentheses.

RNAi constructs	*GPX1*	*GPX2*	*GPX3*	*GPX4*	*GPX5*
RNAi*GPX3* (197nt)	3% (6nt)	1% (2nt)	100% (197nt)	3% (6nt)	3% (6nt)

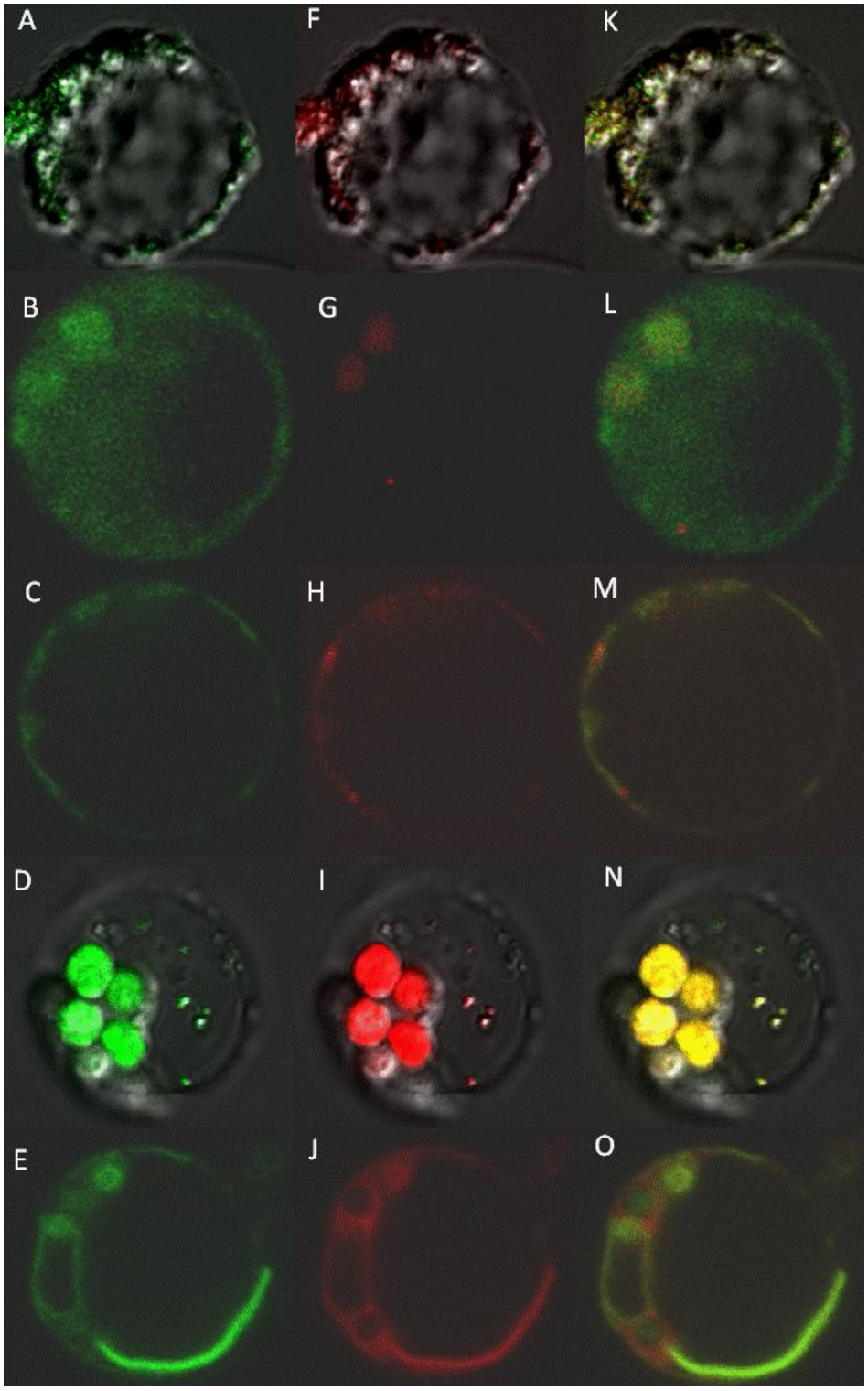


Figure 1. Glutathione peroxidase proteins (OsGPX1, OsGPX2, OsGPX3, OsGPX4 and OsGPX5) are localized in mitochondria, chloroplasts and cytosol. Left panel indicates YFP fluorescence, middle panel indicates chlorophyll auto fluorescence and right panel represents the merged image (left and middle panels). The OsGPX1 (A, F and K) and OsGPX3 (C, H and M) proteins are localized in the mitochondria of rice protoplasts; OsGPX2 (B, G and L) at cytosol and chloroplasts; OsGPX4 (D, I and N) at chloroplasts; OsGPX5 at cytosol/endoplasmic reticulum and chloroplasts (E, J and O).

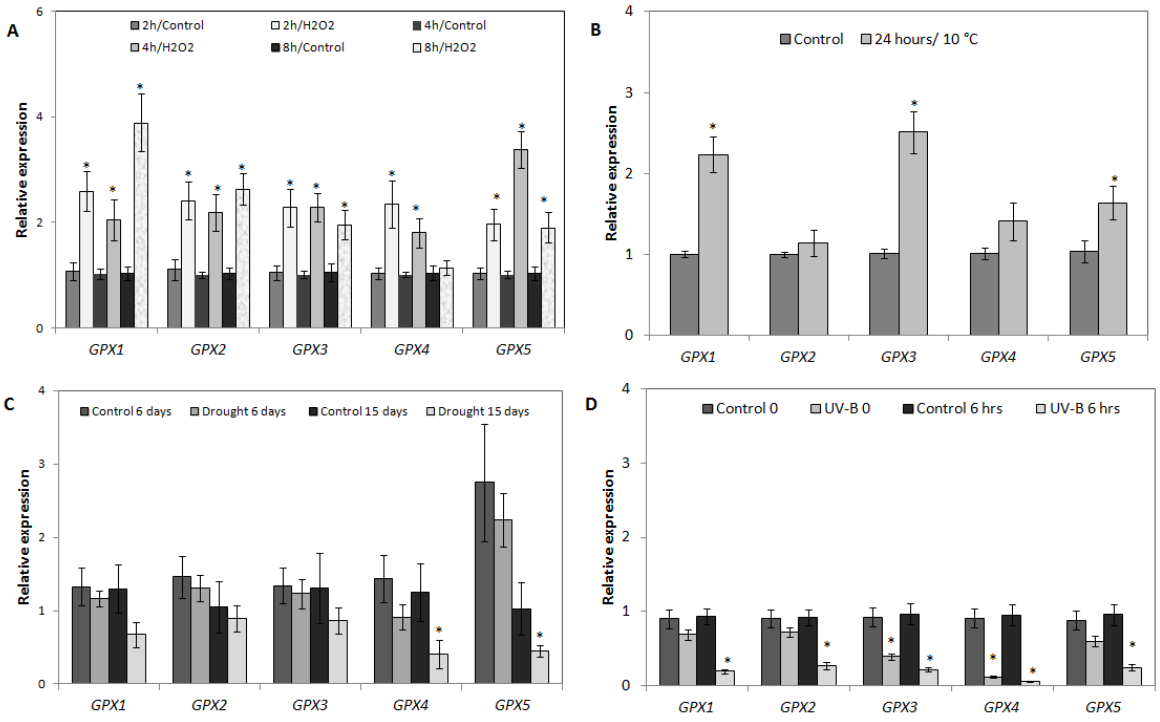


Figure 2. The relative expression of *GPX* genes in rice shoots under different abiotic stress conditions: 10 µM H_2O_2, after 2, 4 and 8 hours of treatment (A); 24 hours at 10 ºC (B); 6 and 15 days without water (C); and immediately (time 0) and 6 hours after 4 hrs of UV-B light treatment. The data represent the means ± SE of three biological replicates. Asterisks indicate significant differences, $p<0.05$.

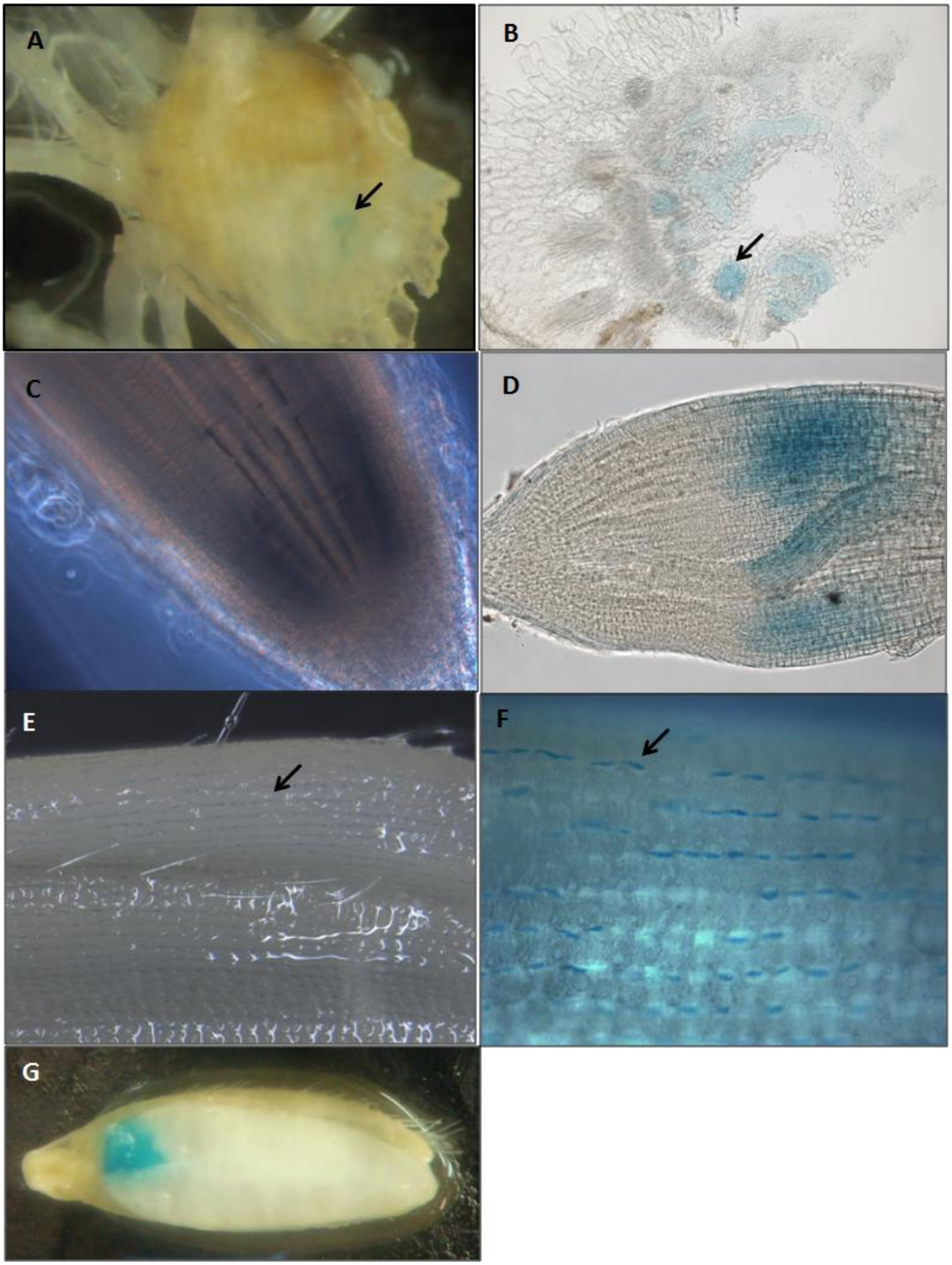


Figure 3. Analysis of the expression patterns of the rice *GPX3* promoter gene merged to the Gus sequence. (A, B) Six-month-old rice plants: phloem of stem and root bases; (C, D) adventicius roots and elongation zone of young root apices; (E, F) young trichomes of rice panicle of 6 months; and (G) embryo of a fully expanded rice seed.

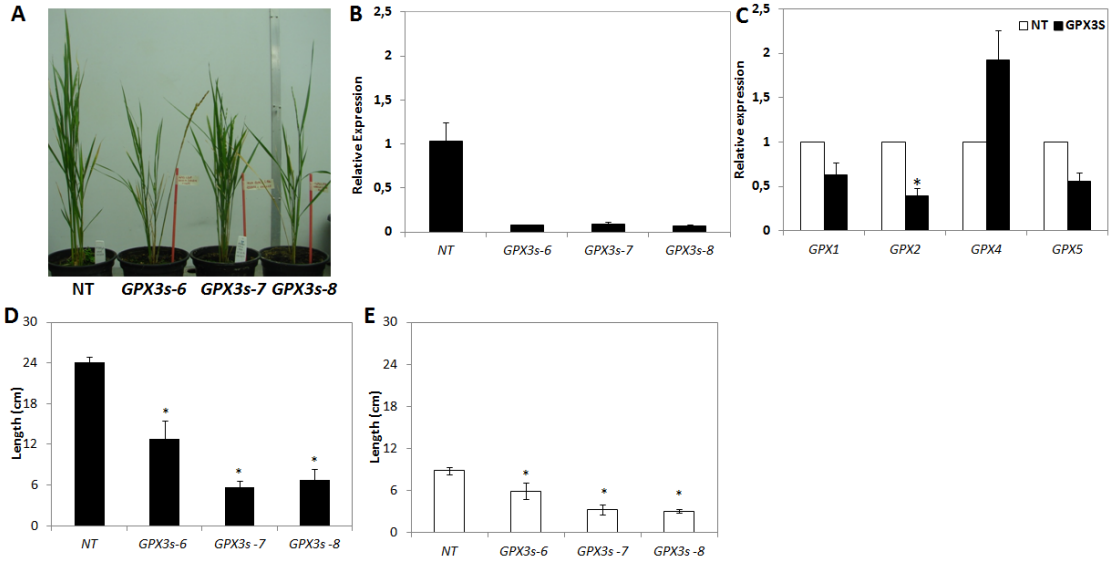


Figure 4. Characterization of GPX3-silenced plants (A-C) for mitochondrial *OsGPX3*. (A) Non-transformed (NT) and three independent GPX3 line plants were grown under controlled conditions. (B) Quantitative determination of *OsGPX3* mRNA in the leaves of NT and *GPX3s* plants grown under control conditions. The transcript level of *OsGPX3* in NT plants was used to normalize transcript accumulation in *GPX3s* and NT plants. (C) Expression analyses of all *OsGPX* genes in *GPX3s* plants compared to NT plants. The values represent the means ± SD (N = 3). Shoot (D) and root (E) length (cm) of 10-day-old rice plants; the values represent the means ± SD (N = 10). Each plant line that promotes a significantly different expression of individual *GPX* compared to NT is designated with an asterisk (*).

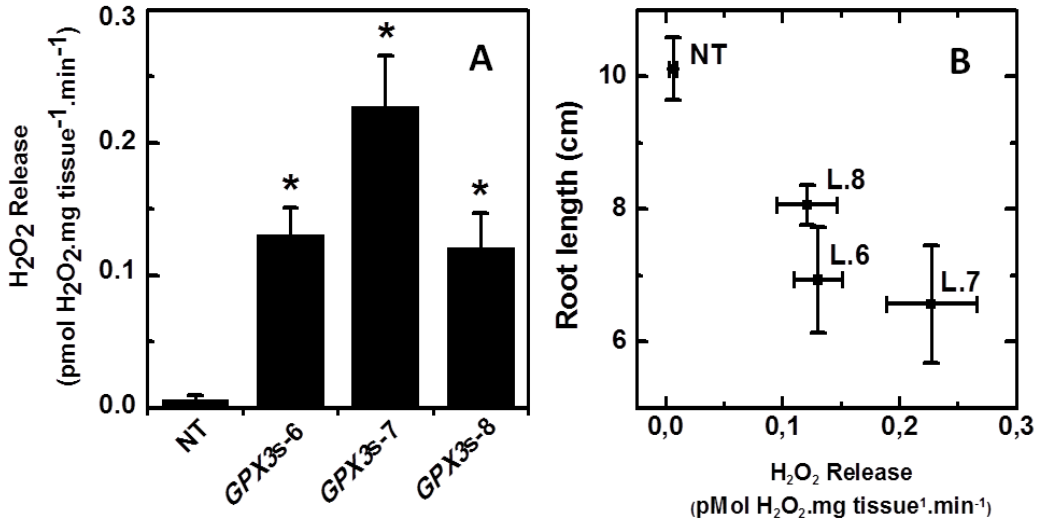
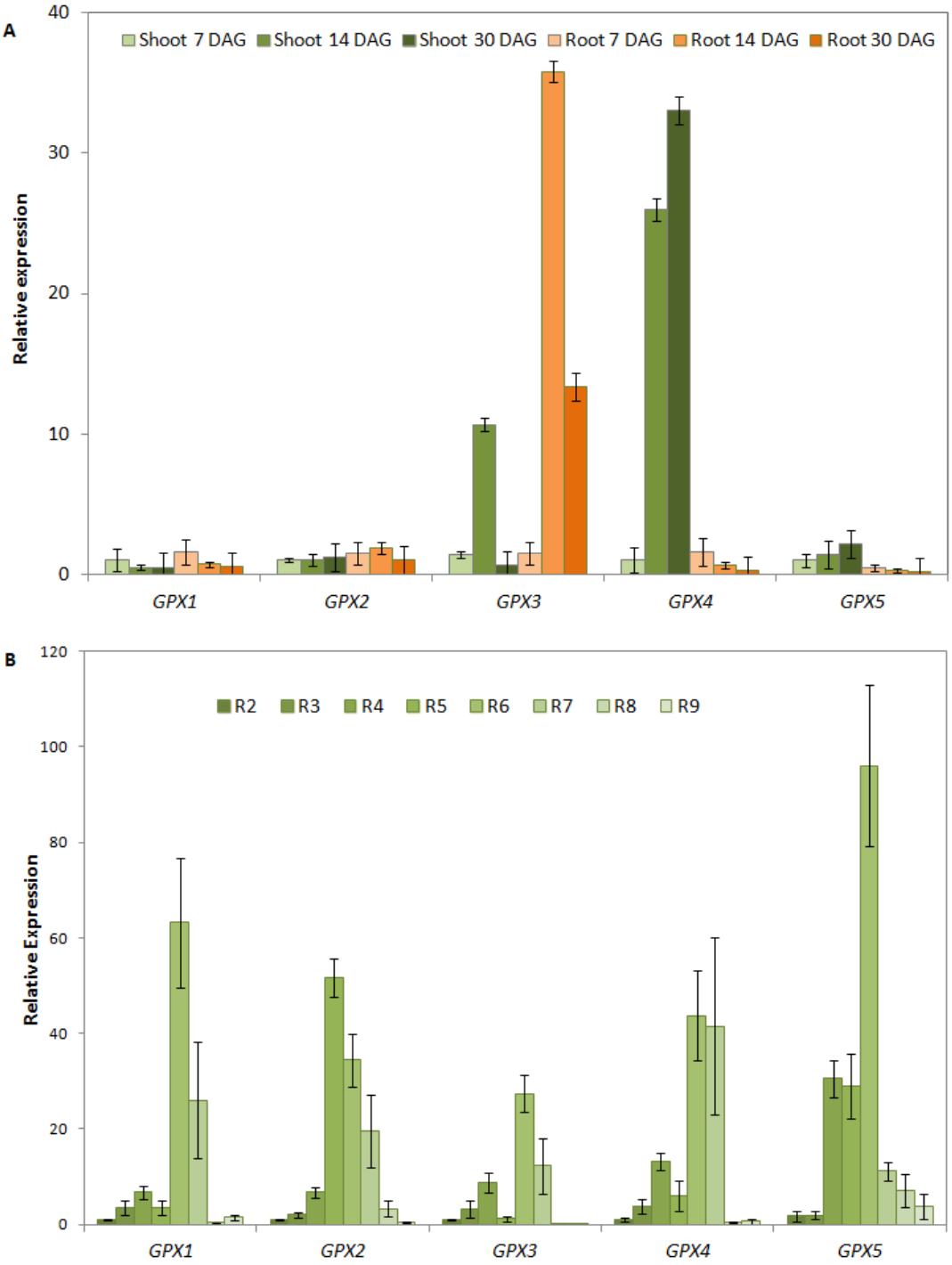
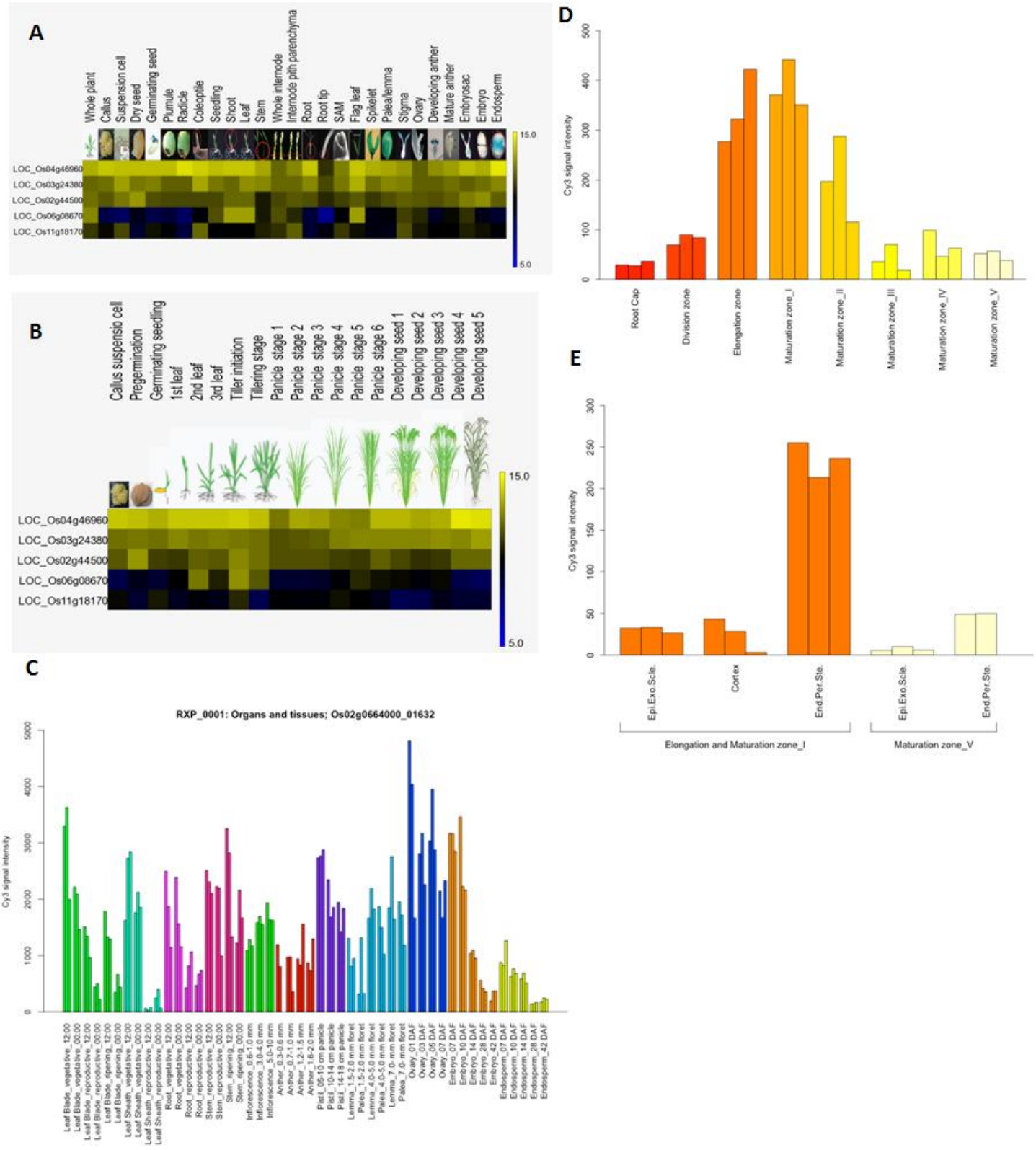


Figure 5. ROS generation in rice roots. The effect of *OsGPX3* silencing was evaluated in three different lines. The release of H_2O_2 was evaluated fluorometrically in three different *GPX3*s plant lines (L-6, 7, and 8) (A), and calculated the ratio between ROS release and root length (B). The values represent the means ± SE of four independent experiments. Asterisks indicate significant differences, $p<0.05$, and the population means are significantly different.



Supplemental Figure 1. The relative expression of rice *GPX* genes in the shoots and roots at 7, 14 and 30 days after germination (DAG, A) and during panicle development stages (R2-R8, B). The data represents the means ± SE of three biological replications.



Supplemental Figure 2. Rice *GPX* gene expression analysis from the Rice Oligonucleotide Array Database in different anatomical parts (A) and during plant development (B). RiceXPro database analysis of different rice organs (C), specifically during root development (D, E). *GPX1* – LOC_Os04g46960, *GPX2* – LOC_Os03g24380, *GPX3* – LOC_Os02g44500, *GPX4* – LOC_Os06g08670, *GPX5* – LOC_Os11g18170).

CAPÍTULO 3

Caracterização fenotípica de plantas de arroz silenciadas para o gene *GPX1* e para *GPX4* .

Com o objetivo de elucidar as funções dos outros membros da família de GPX de arroz, localizados em organelas, utilizamos construções "hairpin" para a isoforma cloroplastídica (*OsGPX4*) e mitocondrial (*OsGPX1*) para a transformação de calos embriogênicos de arroz utilizando *Agrobacterium tumefaciens*. Após várias tentativas de regenerar plantas a partir de calos transformados com a construção *hairpin* para o gene *GPX4* nenhuma planta foi obtida. Os calos transgênicos tornaram-se verdes, mas não foram capazes de emitir radícula ou brotações. Por outro lado, plantas de arroz silenciadas para o gene *GPX1* foram regeneradas com sucesso, no entanto, mostraram fenótipo alterado quando comparado com plantas de arroz não-transformadas.

Os resultados destes estudos resultaram em uma "Brief Communication" intitulada **"Silecing of chloroplastic and mitochondrial-targeted GPX impairs normal plant development in rice"**, submetida para o periódico "*Biologia Plantarum*".

Silecing of chloroplastic and mitochondrial-targeted GPX impairs normal plant development in rice

Gisele Passaia[a], Andréia Caverzan[a, b] Leila Spagnolo Fonini[a, b], Fabricio E. L. Carvalho[c], Joaquim A. Gomes Silveira[c], Márcia Margis-Pinheiro[a, b*]

[a] Department of Genetics, Federal University of Rio Grande do Sul, RS, Brazil.
[b] Biotechnology Center, Federal University of Rio Grande do Sul, RS, Brazil.
[c] Department of Biochemistry and Molecular Biology, Federal University of Ceará, CE, Brazil.

*Corresponding author. Address for correspondence: Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, CEP: 915910-970, Porto Alegre - RS – Brazil, Telephone +55 (51) 3308-9814. E-mail address: marcia.margis@ufrgs.br

Abstract

Plant glutathione peroxidases (GPX) catalyze the reduction of H_2O_2 or organic hydroperoxides to water, mitigating the toxicity of these compounds to the cell. In rice plants, the GPX gene family is composed of five members that are distributed in a range of sub-cellular compartments, including the cytosol, mitochondria, chloroplasts or endoplasmic reticulum. Of these, OsGPX1 and OsGPX4 are located in the mitochondria and the chloroplasts, respectively. To understand the role of these GPX in rice, the effect of the knockdown of *OsGPX1* and *OsGPX4* in rice plants was evaluated. Our data show that OsGPX4 is essential for *in vitro* rice regeneration because no plants were obtained from calli carrying a hairpin construct against *OsGPX4*. Although knockdown of *OsGPX1* did not impair plant regeneration, the plants with silenced *OsGPX1* (*GPX1s* plants) showed a reduced shoot length and reduced number of panicles and seeds compared to the non-transformed (NT) rice plants. These results indicate that OsGPX1 negatively affects shoot and panicle development and OsGPX4 is essential to *in vitro* shoot regeneration.

Key-words: *Oryza sativa*; rice; GPX; glutathione peroxidase; oxidative stress, seed production, *in vitro* regeneration

Reactive oxygen species (ROS), which are generated during normal plant development, act as signaling molecules and regulate essential processes. They are also generated as toxic cellular metabolites, a product of biotic and abiotic stress conditions (Halliwell, 2006; Faltin *et al.*, 2010). To maintain redox homeostasis, ROS-scavenging systems use thiol-containing proteins as redox transducers (Foyer and Noctor, 2005). Plant glutathione peroxidases (GPX) preferentially use thioredoxin as an electron donor to catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols (Herbette *et al.*, 2007). Contrary to most plant GPX, mammalian isoforms carry selenocysteine in their active site, allowing them to preferentially use glutathione as an electron donor (Herbette *et al.*, 2007). Mammalian GPX regulate fundamental cellular processes; for example, the GPX-knockout mouse displays early embryonic lethality (Imai *et al.*, 2003). Additionally, mammalian GPX prevents apoptosis-mediating inflammatory defense mechanisms (Imai Hirotaka, 2003). In plants, GPXs are involved in biotic and abiotic stress responses (Milla *et al.*, 2003; Navrot *et al.*, 2006); for example, in *Arabidopsis thaliana*, GPX loss or gain of function mutants demonstrated the role of these enzymes in H_2O_2 scavenging, signal transduction, photooxidative stress tolerance and nuclear DNA damage protection (Miao et al., 2006; Chang et al., 2009; Gaber et al., 2012). To understand the role of GPX in rice, a monocot plant, we tried to obtain transgenic rice plants that were silenced for the whole GPX family. In the current study, we present the effects of *OsGPX1* and *OsGPX4* knockdown in rice plants. The transformation of rice calli with a hairpin construct targeted to chloroplastic *OsGPX4* failed to regenerate plants; this highlighted the importance of OsGPX4 during *in vitro* regeneration. Moreover, mutants deficient in the mitochondrial *OsGPX1* gene allowed the formation of fertile plants; additionally, these plants (*GPX1s* plants) presented a shorter shoot length compared to non-transformed (NT) plants. However, silencing *OsGPX1* appeared to disturb reproductive processes because the plants exhibited a lower biomass and lower number of panicles and seeds than the NT plants.

Target gene sequences from *OsGPX1* (LOC_Os04g46960) and *OsGPX4* (LOC_Os06g08670) ranging between 115-197 bp were inserted into the pANDA RNAi vector (Miki and Shimamoto, 2004) by the gateway LR reaction, using cDNAs as a template. The following primer pairs were used to amplify the sequences: RNAi*OsGPX1*: 5'-CACCGTCCTCGTCTCCACGCTACC-3' and 5'- AAGTCGTGGACGGAGGTG-3' and RNAi*Os*GPX4: 5'- CACCCTGTTGCAGCATCCTTTTTG-3' and 5'-CTGACGACACCCACAACAAC-3'. The plasmids were introduced into rice calli via *Agrobacterium tumefaciens*-mediated transformation, as described by Upadhyaya, 2002. T1

rice seeds from NT and transgenic plants were germinated in MS medium (Sigma-Aldrich, São Paulo, São Paulo, Brazil) supplemented with hygromycin, under controlled conditions (150 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 25 °C, 80% relative humidity and a 12-h photoperiod). The cDNA and real-time qPCR assays were performed as described by Rosa *et al*. 2010. Primers were designed to produce DNA fragments ranging from 180-250 bp. PCR amplifications were performed using the following specific primers: *OsGPX1*: 5'-AGCAACCTGCACTTATGCACT-3' and 5'- CAGCAAGGAAATTTATTGACATGA-3'; *OsGPX2*: 5'- CTGGTTGGGTAGGCACTGTT-3' and 5'-TGCAAACACAAACCTTACGCTAC-3'; *OsGPX3*: 5'- TTGCATTGAGCACTTGGAAC-3' and 5'- AGGGGCAAAGTGATGCAGTA-3'; *OsGPX4*: 5'-CTGTACATATGCCTTGCCTCA-3' and 5'-GTTACAGGGGCCAGATAAGC-3'; *OsGPX5*: 5'- AAGATTGAGAATGATATCCAGAAGC-3' and 5'-GCAAACCACATTCTTACGAACA-3'. *OsActina2* gene: 5'-GGACGTACAACTGGTATCGTGTT-3' and 5'-GTTCAGCAGTGGTAGTGAAGGAG-3'; *OsFdh3* gene: 5'-TTCCAATGCATTCAAAGCTG-3' and 5'-CAAAATCAGCTGGTGCTTCTC-3'; *OseF1α* gene: 5'-TTTCACTCTTGGTGTGAAGCAGAT-3' and 5'-GACTTCCTTCACGATTTCATCGTAA-3' were used as internal controls to normalize the amount of mRNA present in each sample. All reactions were repeated 4 times, and expression data analyses were performed after comparative quantification of amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). Rice NT and *GPX1s* plants were grown in a greenhouse (mean temperature of 29 °C/24 °C (day/night), 61% mean relative humidity, a 12 h photoperiod and average maximum photosynthetic photon flux density (PPFD) of 850 μmol m^{-2} s^{-1}), until they reached the reproductive phase. Data represent the mean +/- standard error of mean (SEM). Statistical analyses were performed using Student's t-test to compare the mean gene expression values at a confidence level of 0.05. For phenotypic comparisons, data were analyzed using analysis of variance (ANOVA), and the mean values were compared using Tukey´s test at a confidence level of 0.05.

To determine the functional role of mitochondrial *OsGPX1* and chloroplastidial *OsGPX4* in rice, each gene was individually silenced using inverse repeat (IR) constructs transcribing dsRNA (hairpin). We designed an IR construct transcribing dsRNA for a specific region of 115 bp for *OsGPX1* and 197 bp for *OsGPX4*, in the 5'UTR and 3'UTR,

respectively. Rice calli were transformed with the RNAi*OsGPX1* and RNAi*OsGPX4* constructs; while 7 lines carrying the hairpin construct for *OsGPX1* were recovered *(L.4, 17, 29, 30, 36, 40, 45*), all attempts to obtain regenerated plants expressing an RNAi*OsGPX4* construct failed. For this construct, the calli turned green but did not evolve into plants (Fig. 1D). RT-qPCR analysis of *GPX4s* calli showed that the *OsGPX4* mRNA level was reduced to 20 % compared to the NT calli (Fig. 1E). To investigate whether the other *OsGPX* genes could be differently modulated in the *OsGPX4s* calli, we analyzed the transcript levels of the other *OsGPX* genes. The expressions of the cytosolic *OsGPX*2 and *OsGPX*5 genes were reduced to 58 % and 45 %, respectively, while the expressions of the mitochondrial *OsGPX1* and *OsGPX3* genes did not change in the *GPX4s* calli (Fig. 2F). The knockdown of *OsGPX1* produced plants with a shorter shoot length compared to NT plants (Fig. 2A), and RT-qPCR analysis showed that the *OsGPX1* mRNA was reduced to 40 % compared to the NT plants (Fig. 1B). Evaluation of the other members of the family in the *GPX1s* transgenic plants showed a reduction in *OsGPX5* gene expression to 70 % of the NT plants, while the expression of the other genes did not change (Fig. 1C).

Our evaluations illustrated that most of the *GPX1s* plants analyzed had a shorter shoot length compared to the NT plants (Fig. 2A). Although some lines of the *GPX1s* plants had a higher panicle number compared to the NT plants (Fig. 2B), the number of seeds per panicle/plant was lower than in the NT plants (Fig. 2C). A similar trend was observed with the production of viable seeds, *GPX1s* plants had a lower number of viable seeds in the majority of the lines analyzed (Fig. 2D). The viability index of seeds (number of viable seeds over the total number of seeds) followed the same pattern as observed above (Fig. 2E), while the weight of 100 seeds (g) was only different among the NT plants compared with about half the mutant plant lines studied (Fig. 2F).

Our efforts to regenerate the knockdown *GPX4s* rice plants failed on all attempts, although 20 % of the *OsGPX4* expression was retained. It has been reported that the over expression of cit-PHGPX (*Citrus sinensis*) prevented the regeneration of tobacco, potato and tomato plants (Faltin *et al.*, 2010). However, over expression of cit-PHGPX was achieved when tobacco and potato cell cultures were used or the floral-dip method was employed in Arabidopsis plants (Faltin *et al.*, 2010). These data suggest that the equilibrium in H_2O_2 levels, and not merely the presence or absence of H_2O_2, is crucial for *in vitro* regeneration in plants; additionally, the GPX enzymes interfere with shoot organogenesis during this process in different plant species. These data strongly suggest that GPX4 is essential for rice development. It is also possible that the 80 % decrease in the expression of *OsGPX4*, together

with the 42 % and 55 % decrease in *OsGPX2* and *OsGPX5*, respectively, was too drastic for the cells and thus resulted in impaired regeneration. However, the expression of only 40 % *OsGPX1* (Fig. 1B) and 5 % of *OsGPX3* (unpublished results), both mitochondrial GPX isoforms, did not affect *in vitro* regeneration, indicating that reduced expression of mitochondrial GPX does not impair *in vitro* regeneration, but that of chloroplastic GPX4 does.

GPX1s lines expressing only 40 % *OsGPX1* had a shorter shoot length than the NT plants, and their seed production was affected as well. The knockdown of different antioxidant enzymes in rice produces numerous phenotypic alterations, but the reduction in seed production has not yet been described. Previously, our group demonstrated that cytosolic *APX* knockdown in rice plants exhibited a normal phenotype, and photosynthesis parameters were not affected either (Rosa *et al.*, 2010; Ribeiro *et al.*, 2012). Moreover, the reduction in expression of chloroplastic APX in rice did not result in any phenotypic alterations; instead, photosynthesis parameters were affected (Caverzan, data not published).

Taken together, our data demonstrate that the reduced expression of the mitochondrial *OsGPX1* and chloroplastic *OsGPX4* genes in rice affect important processes of rice development, indicating that these enzymes play an essential role in the interaction between redox homeostasis and normal plant development.

Acknowledgments

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and International Centre for Genetic Engineering and Biotechnology (ICGEB).

References

Chang CCC, Slesak I, Jordá L, Sotnikov A, Melzer M, Miszalski Z, Mullineaux PM, Parker JE, Karpinska B, Karpinski S (2009) Arabidopsis chloroplastic glutathione peroxidases play a role in cross talk between photooxidative stress and immune responses. Plant physiology **150**: 670–83

Faltin Z, Holland D, Velcheva M, Tsapovetsky M, Roeckel-Drevet P, Handa AK, Abu-Abied M, Friedman-Einat M, Eshdat Y, Perl A (2010) Glutathione peroxidase regulation of reactive oxygen species level is crucial for in vitro plant differentiation. Plant & cell physiology **51**: 1151–62

Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. The Plant cell **17**: 1866–75

Gaber A, Ogata T, Maruta T, Yoshimura K, Tamoi M, Shigeoka S (2012) The involvement of Arabidopsis glutathione peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. Plant & cell physiology **53**: 1596–606

Herbette S, Roeckel-Drevet P, Drevet JR (2007) Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. The FEBS journal **274**: 2163–80

Imai H, Hirao F, Sakamoto T, Sekine K, Mizukura Y, Saito M, Kitamoto T, Hayasaka M, Hanaoka K, Nakagawa Y (2003) Early embryonic lethality caused by targeted disruption of the mouse PHGPx gene. Biochemical and Biophysical Research Communications **305**: 278–286

Imai Hirotaka IN (2003) Biological significance of phospholipid hydroperoxide glutathione peroxidase (phgpx, gpx4) in mammalian cells. Free Radical Biology & Medicine **34**: 145–169

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) **25**: 402–8

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. The Plant cell **18**: 2749–66

Miki D, Shimamoto K (2004) Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant & cell physiology **45**: 490–5

Milla M a. R, Maurer A, Huete AR, Gustafson JP (2003) Glutathione peroxidase genes in Arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. The Plant Journal **36**: 602–615

Navrot N, Collin V, Gualberto J, Gelhaye E, Hirasawa M, Rey P, Knaff DB, Issakidis E, Jacquot J-P, Rouhier N (2006) Plant glutathione peroxidases are functional peroxiredoxins

distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant physiology **142**: 1364–79

Ribeiro CW, Carvalho FEL, Rosa SB, Alves-Ferreira M, Andrade CMB, Ribeiro-Alves M, Silveira J a G, Margis R, Margis-Pinheiro M (2012) Modulation of genes related to specific metabolic pathways in response to cytosolic ascorbate peroxidase knockdown in rice plants. Plant biology **14**: 944–55

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira J a G, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry **71**: 548–58

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nature Protocols **3**: 1101–1108

Upadhyaya NM, Zhou X-R, Zhu Q-H, Eamens A, Wang M-B, Waterhouse PM, Dennis ES (2002) Transgenic Rice. In Transgenic Cereals, ed O'Brien L , Henry RJ, AACC, Minnesota: 28-87.

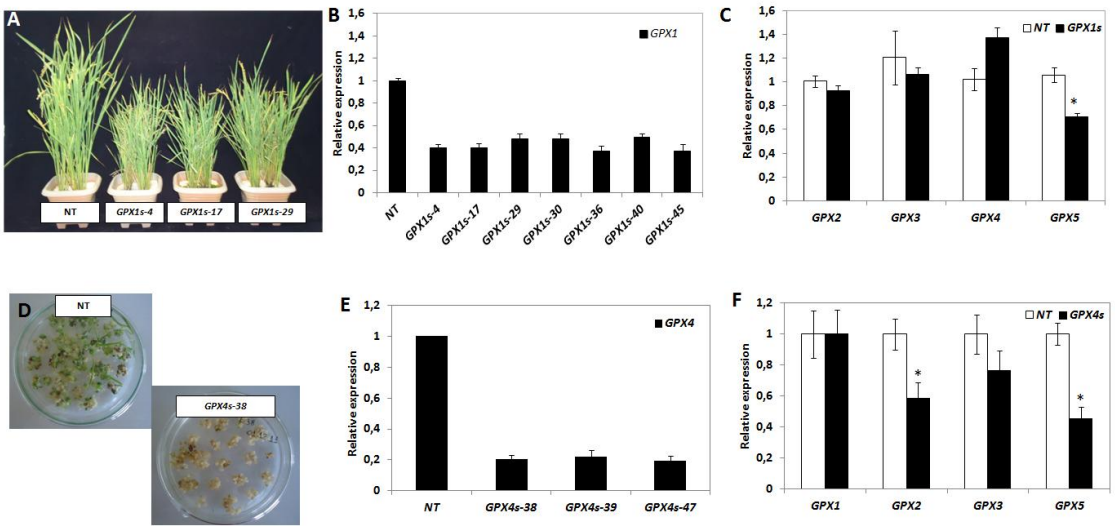


Figure 1. Characterization of *GPX1s* (A-C) and *GPX4s* (D-F) plants with silenced mitochondrial *OsGPX1* and chloroplastidial *OsGPX4*. (A) Non-transformed (NT) and three independent lines of *GPX1s* plants grown under controlled conditions; (B) Quantitative determination of the *GPX1* mRNA in the leaves of the NT and *GPX1s* plants, grown under control conditions. Transcript level of *GPX1* present in the NT plants was used to normalize transcript accumulation in *GPX1s* and NT plants; (C) Expression analyses of all *OsGPX* genes in *GPX1s* plants, in comparison with the expression in NT plants; (D) NT calli and *GPX4s* calli grown in *in vitro* culture; (E) Quantitative determination of the *GPX4* mRNA in leaves of NT and *GPX4s* calli; (F) Expression analysis of all *OsGPX* genes in *GPX4s* calli. Values represent the mean ± SD (N = 3). Each plant line that promotes a significantly different expression of individual *GPX*, compared to NT, was labeled with an asterisk (*).

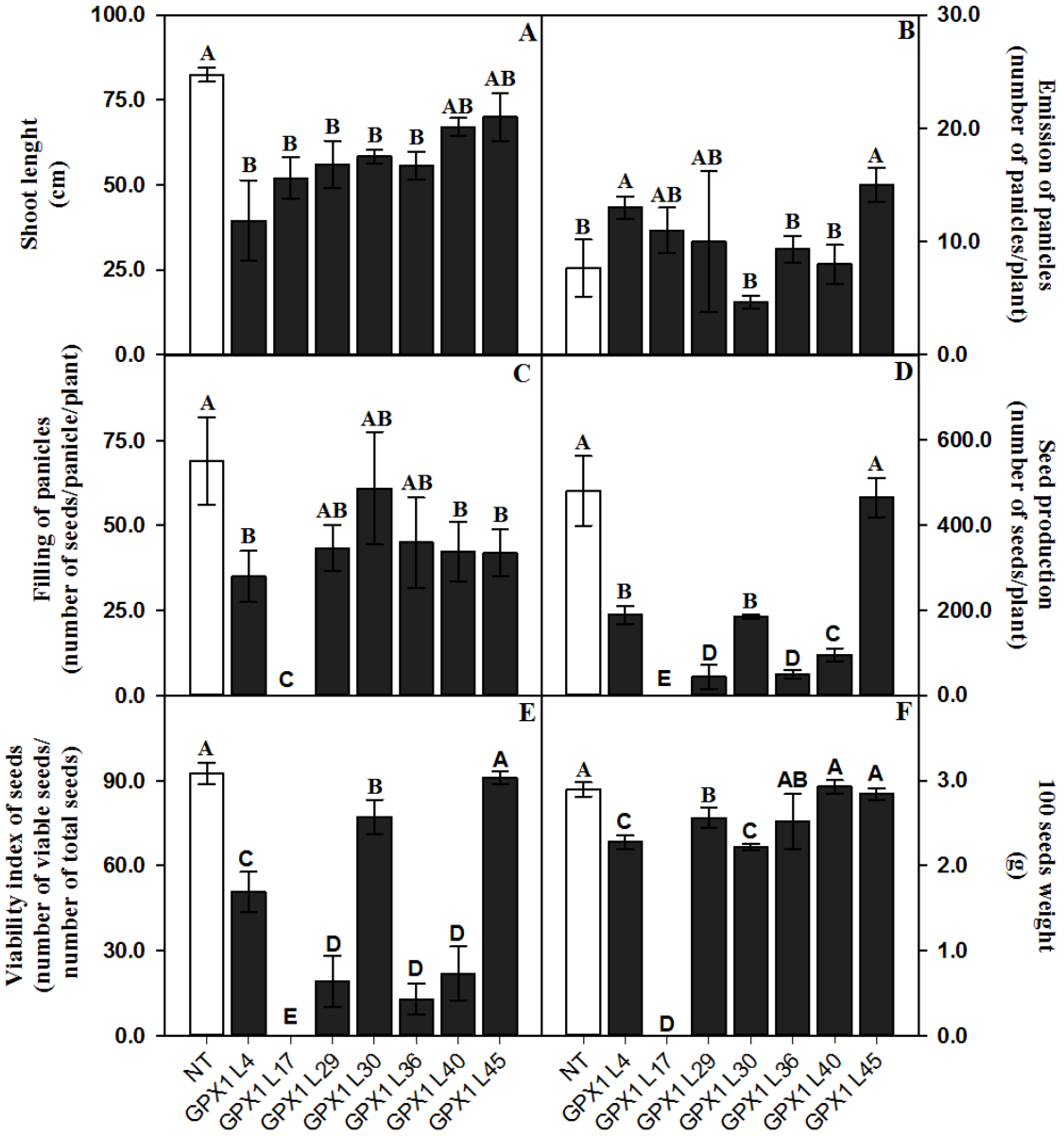


Figure 2: NT and *GPX1s* plants phenology data: height measured from the stem base to the apex of the leaf +1 (A); total panicle number per plant (B); average number of seeds per panicle per plant (C); total number of viable seeds per plant (D); index of viable seeds per panicle (E); weight of 100 seeds (g) (F). Each plant line that presented significantly different results was demarcated with a specific letter (A, B, C or D).

DISCUSSÃO GERAL

Os danos oxidativos derivados das ERO constituem um dos principais fatores de dano para as plantas expostas a estresses ambientais. O sistema detoxificante de ERO mantém a homeostase redox celular em resposta a uma variedade de estresses bióticos e abióticos. Em muitos casos, proteínas que contém tiol servem como transdutores dos sinais redox nas células em resposta a ERO (Foyer and Noctor, 2005a). Os principais candidatos como reguladores de ERO são as proteínas GPX, descritas como sensores redox em levedura (Delaunay et al., 2002) e Arabidopsis (Miao et al., 2006). Utilizando Arabidopsis como modelo de estudo para o entendimento das funções de GPX durante o desenvolvimento da planta e o estabelecimento da arquitetura da raiz dependente de ABA, auxina e SL, mutantes nocaute para sete genes da família de GPX em Arabidopsis foram utilizados (*gpx1*, *gpx2*, *gpx3*, *gpx4*, *gpx6*, *gpx7* e *gpx8*). Foi possível verificar que a expressão de *AtGPX2*, *AtGPX3* e *AtGPX6* é necessária para a formação da raiz dependente de hormônios. O mutante *gpx2* não apresenta estímulo por auxina na fase I do desenvolvimento do primórdio de raízes laterais, sugerindo que o mutante está bloqueado em processos que ocorrem no início do desenvolvimento dessas estruturas. Além disso, o mutante *gpx2* apresentou aumento de inibição de raízes laterais na presença do hormônio SL, indicando que permanece sensível à inibição do transporte de auxina mediado por SL. O mesmo mutante apresentou menor sensibilidade quando tratado com ABA, a diminuição do número de raízes laterais foi menor do que o observado no genótipo selvagem. Por outro lado, mutantes deficientes em *GPX3* apresentaram menor sensibilidade a SL. Por fim, o mutante *gpx6* mostrou maior sensibilidade à ABA e auxina, na presença de ABA apresentando maior inibição do número de raízes laterais do que a planta selvagem, e na presença de auxina produzindo menor número de raízes laterais do que a planta selvagem.

Esses dados indicam que pelo menos três GPX de Arabidopsis são necessárias para a correta formação da arquitetura da raiz dependente dos hormônios ABA, auxina e SL. Cada um dos genes parece desempenhar uma função diferente nesse processo, pois, após a adição do hormônio SL (Figura 8, capítulo 1), por exemplo, os mutantes *gpx2* e *gpx3* apresentaram respostas diferentes. Enquanto o mutante *gpx2* possui menor número de primórdios nos estágios I e II de desenvolvimento do que o tipo selvagem, este permanece sensível à SL, sugerindo que seu sítio de ação é diferente de GPX3. O mutante *gpx3*, por sua vez, apresenta insensibilidade parcial à SL no desenvolvimento

das raízes laterais, sugerindo, que o transporte de auxina já está inibido nesse mutante, mesmo na ausência de SL. Da mesma forma, os mutantes *gpx2* e *gpx6* apresentaram comportamentos diferentes do tipo selvagem quando crescidos na presença de ABA e auxina. O primeiro, mostrando insensibilidade à ABA e maior sensibilidade à auxina, emitindo maior número de raízes laterais do que o tipo selvagem na presença dos dois hormônios. O segundo mostrando maior sensibilidade à ABA do que o tipo selvagem e menos sensibilidade à auxina, emitindo menor número de raízes laterais na presença dos dois hormônios.

O entendimento exato da participação de GPX2, GPX3 e GPX6 no controle da arquitetura das raizes deve ser investigado em maiores detalhes visando estabelecer se a participação dessas proteínas estaria sendo desempenhada na sinalização redox dependente de hormônios, ou se estariam interagindo com outras proteínas que participam do processo de desenvolvimento de raízes laterais. Esse conhecimento é importante para desenvolver plantas mais adaptadas a estresses causados por seca, por exemplo, onde a formação das raízes laterais é importante para a obtenção de nutrientes e água do solo, mesmo em condições desfavoráveis de crescimento.

O estudo da localização subcelular das proteínas de GPX realizado em protoplastos de arroz, confirmou os resultados prévios de estudos *in silico* (Margis et al., 2008) e ainda demonstrou que as proteínas GPX2 e GPX5 estão localizadas em cloroplastos de arroz. Esse duplo direcionamento já foi relatado em arroz para as enzimas APX (Lazzarotto et al., 2011; Caverzan, dados não publicados). Exemplos de proteínas codificadas por um único gene e transportadas para mais de uma organela já são conhecidos (Mackenzie, 2005). Em Arabidopsis os produtos gênicos de ascorbato peroxidase, monodehidroascorbato redutase e glutationa redutase demonstraram duplo direcionamento para plastídeos e mitocôndria, por exemplo (Chew et al., 2003). Proteínas de duplo direcionamento são observadas comumente com tráfego entre mitocôndrias e cloroplastos, o duplo direcionamento também já foi relatado entre mitocôndrias e peroxissomos e ainda entre o núcleo e o citosol. Além disso, o ambiente e o metabolismo da planta influenciam na localização de certas proteínas, mas como isso é determinado, ainda não está completamente esclarecido, mas para algumas proteínas, um dos fatores chave já observados é a luz (Mackenzie, 2005). Por exemplo, o particionamento entre núcleo e citoplasma dos fotoreceptores é influenciado pela luz. A localização subcelular dos fitocromos A, B, C, D e E no núcleo resultam de uma resposta direta à luz (Kircher et al., 2002). O que emerge a partir dos estudos recentes

relacionados ao duplo direcionamento de proteínas, é que as características essenciais para o direcionamento de uma proteína são geralmente mais sutis, facilmente adaptáveis e suscetíveis às influências celulares e ambientais do que se pensava anteriormente (Mackenzie, 2005) . Nossos resultados em protoplastos de arroz também sugerem que os produtos gênicos de *GPX2* e *GPX5* codificam proteínas de duplo direcionamento e que a determinação da localização subcelular destes, pode ser influenciada por diversos fatores ainda não conhecidos e que precisam ser esclarecidos para melhor entender a participação dessas proteínas do sistema antioxidante.

Adicionalmente, níveis de expressão gênica em plantas de arroz expostas a diferentes estresses abióticos e aplicação exógena do H_2O_2 foram determinados (Figura 2, capítulo 2). A expressão de GPX foi induzida fortemente na presença do H_2O_2 exógeno, induzida em nível moderado e apenas para alguns membros da família em condições de baixa temperatura e ainda foram reprimidos ou não modularam em condições de frio, seca, exposição à luz UV-B e presença de alumínio. Ao contrário das APX que são fortemente induzidas em condições de estresses abióticos (Rosa et al., 2010; Bonifacio et al., 2011; Ribeiro et al., 2012, Caverzan et al., dados não publicados). Enquanto em animais as enzimas da família das GPXs são chaves no processo de detoxificação do H_2O_2, em plantas esta função pertence principalmente as catalases e enzimas do ciclo ascorbato-glutationa. A participação de GPX de plantas parece ser mais efetiva quando a maquinaria de detoxificação baseada no ciclo ascorbato-glutationa é exaurida (Gueta-Dahan et al., 1997; Halusková et al., 2009; Bonifacio et al., 2011). Em células de citrus sensíveis ao sal, foi observado o descrécimo da expressão de APX enquanto a atividade de GPX aumentou ao longo do tempo. Além disso, GPXs utilizam hidroperóxidos orgânicos além do H_2O_2 como substrato redutor, estes produtos são gerados especialmente após estresses persistentes e severos gerando a peroxidação lipídica (Gueta-Dahan et al., 1997). Em trabalho anterior, nosso grupo demonstrou que a expressão de GPX de arroz foi induzida em plantas de arroz deficientes nas enzimas ascorbato peroxidase citosólicas em condições normais de crescimento e quando plantas foram submetidas a estresse de calor (Bonifacio et al., 2011). Dessa forma, é possível que em casos de estresses rigorosos, quando os mecanismos antioxidadantes basais estão comprometidos, respostas mais efetivas no combate ao estresse seja assumida por GPXs (Halusková et al., 2009).

Para o estudo funcional dos genes de GPX de arroz, a tecnologia do RNA de interferência foi utilizada. A redução de expressão de três dos cinco membros dessa

família gênica em arroz foi obtida. Todos eles codificam isoformas organelares de GPX. No entanto, a redução da expressão gênica da isoforma cloroplastídica (*GPX4*) em 80% foi letal para o desenvolvimento de plântulas, não permitindo regenerá-las a partir de calos de arroz (Figura 1, capítulo 3). A superexpressão de Cit-PHGPX em tabaco também impediu a regeneração de plantas *in vitro* (Faltin et al., 2010). Por outro lado, o silenciamento individual das isoformas mitocondriais (*GPX1* e *GPX3*), ainda que não tenha impedido a regeneração de plantas, produziu alterações fenotípicas em comparação com o fenótipo normal das plantas NT (Figura 4, capítulo 2; Figura 1 e 2, capítulo 3). As plantas silenciadas *GPX1s* apresentaram menor estatura e apesar de produzirem em média maior número de panículas por planta, o número de sementes viáveis foi inferior ao produzido pelas plantas NT. Esses resultados indicam que o silenciamento do gene *GPX1*, mesmo em condições ideais de crescimento, compromete o crescimento e o desenvolvimento da panícula de arroz. É reconhecido que tanto oxidantes como antioxidantes desempenham papéis na regulação do ciclo celular e alongamento durante o crescimento vegetal. Particularmente os antioxidantes produzidos no apoplasto, ascorbato e monodehidroascorbato possuem funções no controle do alongamento celular através da regulação da rigidez da parede celular, mais ainda, peroxidases e outras enzimas envolvidas na síntese da parede celular também estão envolvidas (Pignocchi and Foyer, 2003). Dessa forma, GPX1 de arroz parece estar envolvida de alguma forma nesses processos celulares, pois a redução de sua expressão também afetou a expressão da isoforma citosólica *GPX5*, o que pode ter levado ao acúmulo exagerado de ERO e consequente retardo de crescimento e afetando o desenvolvimento da panícula.

A redução da expressão de *GPX3* em aproximadamente 97% resultou em um acúmulo de H_2O_2 em raízes cerca de vinte vezes maior do que a planta NT (Figura 5, capítulo 2), gerando plantas que apresentaram raizes e partes aéreas menores em comparação com as plantas NT (Figura 4, capítulo 2). Mutantes de arroz com comprimento das raizes reduzidos já foram descritos na literatura (Ichii M, 1997; Yao et al., 2002; Yao et al., 2004; Jiang et al., 2005), no entanto nenhum deles estaria relacionado com enzimas do sistema antioxidante. Além disso, sabe-se que o crescimento da raiz parece ser fortemente regulado por ERO (Foreman et al., 2003) e glutationa (Vernoux et al., 2000). Adicionalmente, sabe-se que o H_2O_2 inibe o crescimento radicular através da diminuição da expressão de genes relacionados ao ciclo celular (Tsukagoshi, 2012). O hormônio auxina é conhecido como regulador

importante do crescimento da raiz, acumulando no lado inferior ao que recebe o estímulo gravitrópico inibindo crescimento e produzindo ERO (Schopfer et al., 2002). Portanto pode-se estabelecer uma relação entre o acúmulo exagerado no H_2O_2 nas plantas *GPX3s* e a inibição do alongamento dar raiz, resultado da redução dos níveis da proteína GPX3. A análise da atividade da região promotora de *GPX3* fusionado à *Gus* mostrou marcação na região do floema da base do caule e ápice de raízes jovens (Figura 3, capítulo 2), o que reforça a hipótese de participação dessa proteína no desenvolvimento desse órgão. A expressão de *Gus* também foi encontrada em tricomas jovens e no embrião de sementes maduras. As gramíneas possuem formação anatômica complexa do embrião, e vários órgãos embrião-específico estão presentes, entre eles primórdios foliares e radícula (Itoh et al., 2005). Dessa forma, a presença de GPX3 nessa estrutura indica participação importante no início do desenvolvimento da planta de arroz.

A Figura 3 mostra um desenho esquemático da participação de GPX1, GPX3 e GPX4 durante o desenvolvimento vegetativo e reprodutivo da planta de arroz resultantes desse trabalho.

Trabalhos futuros são necessários para estabelecer como as GPX interagem com hormônios e o metabolismo redox controlando o crescimento celular. Questões importantes ainda precisam ser respondidas. Como as várias vias de percepção de ERO, entre as quais se incluem as proteínas GPX, interagem com o H_2O_2 e transmitem o sinal para outras vias de sinalização ainda não é totalmente compreendido. Não há dúvidas que a sinalização redox é central no controle dos mecanismos pelos quais as plantas percebem o ambiente e fazem os ajustes apropriados de expressão gênica, metabolismo e fisiologia (Foyer and Noctor, 2005b) e estudos nessa área de conhecimento são necessários. O entendimento dessas vias de sinalização pode contribuir para o desenvolvimento de estratégias visando à obtenção de plantas mais adaptadas ao ambiente e com maior produtividade.

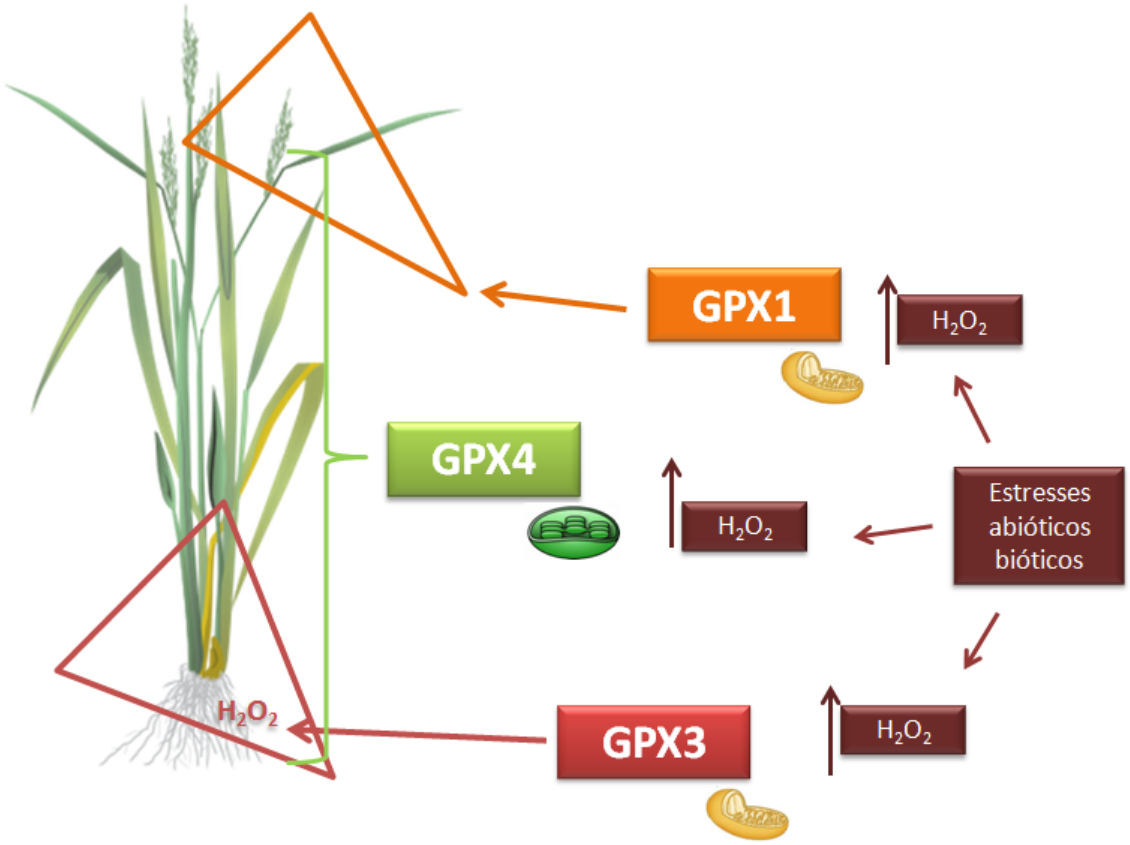


Figura 3. Representação esquemática da participação de GPX1, GPX3 e GPX4 para o desenvolvimento de plantas de arroz. OsGPX são altamente induzidos pela presença do H_2O_2, o qual, é induzido por estresses bióticos e abióticos. GPX1 e GPX3, isoformas mitocondriais, participam da correta formação da raíz, parte aérea e panículas, a presença de GPX3 nas raizes mantém os níveis de H_2O_2 equilibrados. Enquanto que GPX4, isoforma exclusivamente cloroplastídica, é essencial para a regeneração da planta cultivada *in vitro*.

REFERÊNCIAS BIBLIOGRÁFICAS

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science (New York, NY) **301**: 653–7

Azpiroz-Leehan, R., and Feldmann KA (1997) T-DNA insertion mutagenesis in Arabidopsis: Going back and forth. Trends Genet **13**: 152–159

Bartoli CG, Casalongué C a., Simontacchi M, Marquez-Garcia B, Foyer CH (2012) Interactions between hormone and redox signalling pathways in the control of growth and cross tolerance to stress. Environmental and Experimental Botany. doi: 10.1016/j.envexpbot.2012.05.003

Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld J-P (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in Arabidopsis auxin signaling. The Plant cell **22**: 376–91

Bonifacio A, Martins MO, Ribeiro CW, Fontenele A V, Carvalho FEL, Margis-Pinheiro M, Silveira J a G (2011) Role of peroxidases in the compensation of cytosolic ascorbate peroxidase knockdown in rice plants under abiotic stress. Plant, cell & environment **34**: 1705–22

Casimiro I, Marchant a, Bhalerao RP, Beeckman T, Dhooge S, Swarup R, Graham N, Inzé D, Sandberg G, Casero PJ, et al (2001) Auxin transport promotes Arabidopsis lateral root initiation. The Plant cell **13**: 843–52

Chandrasekar V (2008) Physiological and biochemical responses of hexaploid and tetraploid wheat to drought stress. Journal of Agronomy **074**: 108–116

Chang CCC, Slesak I, Jordá L, Sotnikov A, Melzer M, Miszalski Z, Mullineaux PM, Parker JE, Karpinska B, Karpinski S (2009) Arabidopsis chloroplastic glutathione peroxidases play a role in cross talk between photooxidative stress and immune responses. Plant physiology **150**: 670–83

Chew O, Whelan J, Millar a H (2003) Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. The Journal of biological chemistry **278**: 46869–77

Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB (2002) A thiol peroxidase is an H2O2 receptor and redox-transducer in gene activation. Cell **111**: 471–81

Dietz K, Jacob S, Oelze M, Laxa M, Tognetti V, Marina S, Miranda N De, Baier M, Finkemeier I (2006) The function of peroxiredoxins in plant organelle redox metabolism. **57**: 1697–1709

Dietz K-J (2008) Redox signal integration: from stimulus to networks and genes. Physiologia plantarum **133**: 459–68

Domagalska M a, Leyser O (2011) Signal integration in the control of shoot branching. Nature reviews Molecular cell biology **12**: 211–21

Espinoza SE, Guo H, Fedarko N, Dezern A, Fried LP, Xue Q, Beamer B, Walston JD (2010) Glutathione Peroxidase Enzyme Activity in Aging. J Gerontol A Biol Sci Med Sci **63**: 505–509

Faltin Z, Holland D, Velcheva M, Tsapovetsky M, Roeckel-Drevet P, Handa AK, Abu-Abied M, Friedman-Einat M, Eshdat Y, Perl A (2010) Glutathione peroxidase regulation of reactive oxygen species level is crucial for in vitro plant differentiation. Plant & cell physiology **51**: 1151–62

Finnegan PM, Soole KL, Umbach AL. 2004. Alternative mitochondrial electron transport proteins in higher plants. In Advances inPhotosynthesis andRespiration, ed.DADay,AHMillar, JWhelan. Boston: Kluwer. In press

Foreman J, Demidchik V, Bothwell JHF, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JDG, et al (2003) Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422**: 442–6

Foyer and Noctor (2009) Redox Regulation in Photosynthetic Organisms: Signaling, Acclimation, and Practical Implications. Antioxidants & redox signaling **11**: 861–905

Foyer CH, Noctor G (2011) Ascorbate and glutathione: the heart of the redox hub. Plant physiology **155**: 2–18

Foyer CH, Noctor G (2005a) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. The Plant cell **17**: 1866–75

Foyer CH, Noctor G (2005b) Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant, Cell and Environment **28**: 1056–1071

Gaber A, Ogata T, Maruta T, Yoshimura K, Tamoi M, Shigeoka S (2012) The involvement of Arabidopsis glutathione peroxidase 8 in the suppression of oxidative damage in the nucleus and cytosol. Plant & cell physiology **53**: 1596–606

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **48**: 909–30

Gomes & Magalhães Jr. (Organizadores) Arroz irrigado no Sul do Brasil. Brasília, DF. Embrapa Informações Tecnológicas, 2004. 899p

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun E a, Pillot J-P, Letisse F, Matusova R, Danoun S, Portais J-C, et al (2008) Strigolactone inhibition of shoot branching. Nature **455**: 189–94

Guan LM, Zhao J, Scandalios JG (2000) Cis-elements and trans-factors that regulate expression of the maize Cat1 antioxidant gene in response to ABA and osmotic stress: H2O2 is the likely intermediary signaling molecule for the response. The Plant journal : for cell and molecular biology **22**: 87–95

Gueta-Dahan Y, Yaniv Z, Zilinskas B a, Ben-Hayyim G (1997) Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in citrus. Planta **203**: 460–9

Halusková L., Valentovicová K., Huttová J., Mistrík LT (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. Plant Physiology and Biochemistry **47**: 1069–1074

Herbette S, Roeckel-Drevet P, Drevet JR (2007) Seleno-independent glutathione peroxidases. More than simple antioxidant scavengers. The FEBS journal **274**: 2163–80

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. The Plant journal : for cell and molecular biology **6**: 271–82

Hourton-Cabassa C, Rita Matos A, Zachowski A, Moreau F (2004) The plant uncoupling protein homologues: a new family of energy-dissipating proteins in plant mitochondria. Plant physiology and biochemistry **42**: 283–90

Hu X, Bidney DL, Yalpani N, Duvick JP, Crasta O, Folkerts O, Lu G (2003) Overexpression of a Gene Encoding Hydrogen Peroxide- Generating Oxalate Oxidase Evokes Defense Responses in Sunflower 1. **133**: 170–181

Ichii M IM (1997) Genetic analysis of newly induced short-root mutant in rice (Oryza sativa L.). Breeding Science **47**: 121–125

Itoh J-I, Nonomura K-I, Ikeda K, Yamaki S, Inukai Y, Yamagishi H, Kitano H, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant & cell physiology **46**: 23–47

Jiang H, Wang S, Dang L, Wang S, Chen H, Wu Y (2005) A Novel Short-Root Gene Encodes a Glucosamine-6-Phosphate Acetyltransferase Required for Maintaining Normal Root Cell Shape in Rice 1. Plant physiology **138**: 232–242

Joo JH, Bae YS, Lee JS (2001) Role of Auxin-Induced Reactive Oxygen Species in. Plant Physiology **126**: 1055–1060

Kerchev PI, Pellny TK, Vivancos PD, Kiddle G, Hedden P, Driscoll S, Vanacker H, Verrier P, Hancock RD, Foyer CH (2011) The transcription factor ABI4 Is

required for the ascorbic acid-dependent regulation of growth and regulation of jasmonate-dependent defense signaling pathways in Arabidopsis. The Plant cell **23**: 3319–34

Kiddle G, Pastori GM, Bernard S, Pignocchi C, Antoniw J, Verrier PJ, Foyer CH (2003) Effects of leaf ascorbate content on defense and photosynthesis gene expression in Arabidopsis thaliana. Antioxidants & redox signaling **5**: 23–32

Kircher S, Gil P, Kozma-bognár L, Fejes E, Speth V, Husselstein-muller T, Bauer D, Ádám É, Schäfer E, Nagy F (2002) Nucleocytoplasmic Partitioning of the Plant Photoreceptors Phytochrome A , B , C , D , and E Is Regulated Differentially by Light and Exhibits a Diurnal Rhythm. The Plant cell **14**: 1541–1555

Koprivova A, Mugford ST, Kopriva S (2010) Arabidopsis root growth dependence on glutathione is linked to auxin transport. Plant cell reports **29**: 1157–67

Krysan, P.J., Young, J.C., and Sussman MR (1999) T-DNA as an insertional mutagen in Arabidopsis. Plant Cell **11**: 2283–2290

Lazzarotto F, Teixeira FK, Rosa SB, Dunand C, Fernandes CL, Fontenele ADV, Silveira JAG, Verli H, Margis R, Margis-Pinheiro M (2011) Ascorbate peroxidase-related (APx-R) is a new heme-containing protein functionally associated with ascorbate peroxidase but evolutionarily divergent. The New phytologist **191**: 234–50

Leyser O (2005) Auxin distribution and plant pattern formation: how many angels can dance on the point of PIN? Cell **121**: 819–22

Mackenzie S a (2005) Plant organellar protein targeting: a traffic plan still under construction. Trends in cell biology **15**: 548–54

Margis R, Dunand C, Teixeira FK, Margis-Pinheiro M (2008) Glutathione peroxidase family - an evolutionary overview. The FEBS journal **275**: 3959–70

Méplan C, Crosley LK, Nicol F, Horgan GW, Mathers JC, Arthur JR, Hesketh JE (2008) Functional effects of a common single-nucleotide polymorphism (GPX4c718t) in the glutathione peroxidase 4 gene: interaction with sex. The American journal of clinical nutrition **87**: 1019–27

Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. The Plant cell **18**: 2749–66

Milla M a. R, Maurer A, Huete AR, Gustafson JP (2003) Glutathione peroxidase genes in Arabidopsis are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. The Plant Journal **36**: 602–615

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends in plant science **9**: 490–8

Møller IM (2001) Plant mitochondria and oxidative stress : Electron Transport , NADPH Turnover , and Metabolism of Reactive Oxygen Species. Annu Rev Plant Physiol **52**: 561–591

Møller IM, Sweetlove LJ (2010) ROS signalling--specificity is required. Trends in plant science **15**: 370–4

Moore, K.M. Devo, Z. Wang MDG (1995) Cereal genome evolution. Grasses, line up and form a circle. Curr Biol **5**: 737–739

O'Kane SL, Al-Shehbaz I a. (1997) A Synopsis of Arabidopsis (Brassicaceae). Novon **7**: 323

Parinov S, Sevugan M, Ye D, Yang WC, Kumaran M, Sundaresan V (1999) Analysis of flanking sequences from dissociation insertion lines: a database for reverse genetics in Arabidopsis. The Plant cell **11**: 2263–70

Pastori GM, Kiddle G, Antoniw J, Bernard S, Veljovic-jovanovic S, Verrier PJ, Noctor G, Foyer CH (2003) Leaf Vitamin C Contents Modulate Plant Defense Transcripts and Regulate Genes That Control Development through Hormone Signaling. The Plant cell **15**: 939–951

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. Nature **406**: 731–4

Pignocchi C, Foyer CH (2003) Apoplastic ascorbate metabolism and its role in the regulation of cell signalling. Current Opinion in Plant Biology **6**: 379–389

Pignocchi C., Kiddle G., Hernández I., Foster S.J., Asensi A., JB T.T. and CHF (2006) Ascorbate Oxidase-Dependent Changes in the Redox State of the Apoplast Modulate Gene Transcript Accumulation Leading to Modified Hormone Signaling and Orchestration of Defense Processes in Tobacco. Plant physiology **141**: 423–435

Potters G, Horemans N, Jansen M a K (2010) The cellular redox state in plant stress biology--a charging concept. Plant physiology and biochemistry : PPB / Société française de physiologie végétale **48**: 292–300

Potters G, Pasternak TP, Guisez Y, Jansen M a K (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant, cell & environment **32**: 158–69

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen M a K (2007) Stress-induced morphogenic responses: growing out of trouble? Trends in plant science **12**: 98–105

Puntarulo S, Sánchez R a, Boveris a (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant physiology **86**: 626–30

Quan L-J, Zhang B, Shi W-W, Li H-Y (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. Journal of integrative plant biology **50**: 2–18

Rasmusson AG, Soole KL, Elthon TE (2004) Alternative NAD(P)H dehydrogenases of plant mitochondria. Annual review of plant biology **55**: 23–39

Raven, P.; Evert, R., Eichhorn, S. 2007. Biologia Vegetal. Ed.Guanabara. Koogan.

Rhoads DM, Umbach AL, Subbaiah CC, Siedow JN (2006) Mitochondrial Reactive Oxygen Species . Contribution to Oxidative Stress and Interorganellar Signaling. **141**: 357–366

Ribeiro CW, Carvalho FEL, Rosa SB, Alves-Ferreira M, Andrade CMB, Ribeiro-Alves M, Silveira J a G, Margis R, Margis-Pinheiro M (2012) Modulation of genes related to specific metabolic pathways in response to cytosolic ascorbate peroxidase knockdown in rice plants. Plant biology **14**: 944–55

Rosa SB, Caverzan A, Teixeira FK, Lazzarotto F, Silveira J a G, Ferreira-Silva SL, Abreu-Neto J, Margis R, Margis-Pinheiro M (2010) Cytosolic APx knockdown indicates an ambiguous redox responses in rice. Phytochemistry **71**: 548–58

Ruyter-Spira C, Kohlen W, Charnikhova T, Van Zeijl A, Van Bezouwen L, De Ruijter N, Cardoso C, Lopez-Raez JA, Matusova R, Bours R, et al (2011) Physiological effects of the synthetic strigolactone analog GR24 on root system architecture in Arabidopsis: another belowground role for strigolactones? Plant physiology **155**: 721–34

Schopfer P, Liszkay A, Bechtold M, Frahry G, Wagner A (2002) Evidence that hydroxyl radicals mediate auxin-induced extension growth. Planta **214**: 821–8

Schopfer P, Plachy C, Frahry G (2001) Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant physiology **125**: 1591–602

Schwartz SH, Qin X, Zeevaart JAD (2003) Elucidation of the Indirect Pathway of Abscisic Acid Biosynthesis by Mutants , Genes , and Enzymes. Plant physiology **131**: 1591–1601

Sessions A, Burke E, Presting G, Aux G, Mcelver J, Patton D, Dietrich B, Ho P, Bacwaden J, Ko C, et al (2002) GENOMICS ARTICLE A High-Throughput Arabidopsis Reverse Genetics System. **14**: 2985–2994

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and function of ascorbate peroxidase isoenzymes. Journal of experimental botany **53**: 1305–19

Song X.G., She X.P., He J.M. HC and STS (2006) Cytokinin- and auxin-induced stomatal opening involves a decrease in levels of hydrogen peroxide in guard cells of Vicia faba. Functional Plant Biology **33**: 573–583

Speulman E, Metz PLJ, Arkel G Van, Hekkert L, Stiekema WJ, Pereira A (1999) GENOMICS ARTICLE A Two-Component Enhancer-Inhibitor Transposon Mutagenesis System for Functional Analysis of the Arabidopsis Genome. **11**: 1853–1866

Steve L. O'Kane J and IAA-S (2003) Position and generic limits of arabidopsis based on sequences of nuclear ribosomal. Botanical, Missouri Press, Garden Garden, Missouri Botanical **90**: 603–612

Stirnberg P, Van De Sande K, Leyser HMO (2002) MAX1 and MAX2 control shoot lateral branching in Arabidopsis. Development (Cambridge, England) **129**: 1131–41

Sussman MR, Amasino RM, Young JC, Krysan PJ, Austin-phillips S (2000) Resources and Opportunities The Arabidopsis Knockout Facility at the University of Wisconsin – Madison 1. **124**: 1465–1467

Tissier a F, Marillonnet S, Klimyuk V, Patel K, Torres M a, Murphy G, Jones JD (1999) Multiple independent defective suppressor-mutator transposon insertions in Arabidopsis: a tool for functional genomics. The Plant cell **11**: 1841–52

Tognetti VB, Mühlenbock P, Van Breusegem F (2012) Stress homeostasis - the redox and auxin perspective. Plant, cell & environment **35**: 321–33

Toppo S, Flohé L, Ursini F, Vanin S, Maiorino M (2009) Catalytic mechanisms and specificities of glutathione peroxidases: variations of a basic scheme. Biochimica et biophysica acta **1790**: 1486–500

Toppo S, Vanin S, Bosello V, Tosatto SCE (2008) Evolutionary and structural insights into the multifaceted glutathione peroxidase (Gpx) superfamily. Antioxidants & redox signaling **10**: 1501–14

Tsukagoshi H (2012) Defective root growth triggered by oxidative stress is controlled through the expression of cell cycle-related genes. Plant science **197**: 30–9

Tyburski J, Krzemiński Ł, Tretyn A (2007) Exogenous auxin affects ascorbate metabolism in roots of tomato seedlings. Plant Growth Regulation **54**: 203–215

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda-Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K, et al (2008) Inhibition of shoot branching by new terpenoid plant hormones. Nature **455**: 195–200

Urban P, Mignotte C, Kazmaier M, Delorme F, Pompon D (1997) Cloning, yeast expression, and characterization of the coupling of two distantly related Arabidopsis thaliana NADPH-cytochrome P450 reductases with P450 CYP73A5. The Journal of biological chemistry **272**: 19176–86

Vernoux T, Wilson RC, Seeley K a, Reichheld JP, Muroy S, Brown S, Maughan SC, Cobbett CS, Van Montagu M, Inzé D, et al (2000) The root meristemless1/cadmium sensitive2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. The Plant cell **12**: 97–110

Vij S, Gupta V, Kumar D, Vydianathan R, Raghuvanshi S, Khurana P, Khurana JP, Tyagi AK (2006) Decoding the rice genome. BioEssays : news and reviews in molecular, cellular and developmental biology **28**: 421–32

Vranová E, Atichartpongkul S, Villarroel R, Van Montagu M, Inzé D, Van Camp W (2002) Comprehensive analysis of gene expression in Nicotiana tabacum leaves acclimated to oxidative stress. Proceedings of the National Academy of Sciences of the United States of America **99**: 10870–5

Walters DR (2003) Polyamines and plant disease. Phytochemistry **64**: 97–107

Woo HR, Chung KM, Park JH, Oh S a, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. The Plant cell **13**: 1779–90

Xu W, Jia L, Shi W, Liang J, Zhou F, Li Q, Zhang J (2012) Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. The New phytologist. doi: 10.1111/nph.12004

Yao S-G, Mushika J, Taketa S, Ichii M (2004) The short–root mutation srt5 defines a sugar-mediated root growth in rice (Oryza sativa L.). Plant Science **167**: 49–54

Yao S-G, Taketa S, Ichii M (2002) A novel short-root gene that affects specifically early root development in rice (Oryza sativa L.). Plant Science **163**: 207–215

Zhang X, Zhang L, Dong F, Gao J, Galbraith DW, Song CP (2001) Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in Vicia faba. Plant physiology **126**: 1438–48

http://rgp.dna.affrc.go.jp/IRGSP/

http://www.agencia.cnptia.embrapa.br

http://www.faostat.fao.org

CURRICULUM VITAE

Dados pessoais

Nome Gisele Passaia
Nascimento 23/06/1982 - Bento Gonçalves/RS - Brasil
CPF 002.031.400-01

Formação acadêmica/titulação

2009 Ingresso no Doutorado em Genética e Biologia Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
Título: Caracterização funcional de genes de Glutationa peroxidase (GPX)
Orientador: Márcia N.P.Margis
Bolsista do (a): Conselho Nacional de Desenvolvimento Científico e Tecnológico

2007 - 2009 Mestrado em Biologia Celular e Molecular.
Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, Brasil.
Título: Caracterização do perfil de genes diferencial, Ano de obtenção: 2009.
Orientador: Dra. Márcia Pinheiro Margis
Bolsista do (a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

2000 - 2006 Graduação em Ciências Biológicas - Bacharelado.
Universidade do Vale do Rio dos Sinos, UNISINOS, Sao Leopoldo, Brasil.
Título: Caracterização do perfil de genes diferencialmente regulados durante o desenvolvimento do fruto nas cultivares Isabel e Isabel Precoce
Orientador: Dr. Luis Fernando Revers

2000 - 2006 Graduação em Ciências Biológicas - Licenciatura.
Universidade do Vale do Rio dos Sinos, UNISINOS, Sao Leopoldo, Brasil.

Formação complementar

2008 - 2008 Curso de curta duração em Noções e Aplicações em Bioinformática.
EMBRAPA Trigo, CNPT, Brasil.

2008 - 2008 Curso de curta duração em MicroRNAs aplicado ao estudo de processos biológicos.
54º Congresso Brasileiro de Genética, SBG, Brasil.

2006 - 2006 Curso de curta duração.
52º Congresso Brasileiro de Genética, SBG, Brasil.

2006 - 2006 Curso de curta duração em Tratamento de Águas e Efluentes.
Universidade Luterana do Brasil, ULBRA, Canoas, Brasil.

Atuação profissional

1. EMBRAPA Uva e Vinho - CNPUV

Vínculo institucional

2009 - Atual Vínculo: Bolsa de doutorado, Enquadramento funcional: Estudante de doutorado, Carga horária: 40 Regime: Integral

2007 - 2009 Vínculo: Bolsa de mestrado, Enquadramento funcional: Estudante de mestrado, Carga horária: 40, Regime: Integral

2005 - 2007 Vínculo: Bolsa de Iniciação Científica, Enquadramento funcional: Pesquisa em gênomica da videira, Carga horária: 20, Regime: Parcial

---

Atividades

01/2005 - Atual Estágio, Laboratório de Biologia Molecular Vegetal.
Estágio:
Núcleo de pesquisa em genômica da videira.

Prêmios e títulos

2011 Prêmio Pós-graduação - Oral de melhor trabalho na área de Genética, Evolução e Melhoramento de Plantas, 57º Congresso Brasileiro de Genética.

Producão

Produção bibliográfica

Artigos completos publicados em periódicos

1. FAWAL, N., LI, Q., SAVELLI, B., BRETTE, M., **PASSAIA, G.**, FABRE, M., MATHE, C., DUNAND, C.
PeroxiBase: a database for large-scale evolutionary analysis of peroxidases. Nucleic Acids Research (Online). , v.40, p.1 - 4, 2012.

2. CAVERZAN, A., **PASSAIA, G.**, ROSA, S. B., RIBEIRO, C. W., LAZAROTTO, F., MARGIS-PINHEIRO, M.
Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. Genetics and Molecular Biology (Impresso). , v.35, p.1011 - 1019, 2012.

3. COSTENARO-DA-SILVA, D., **PASSAIA, G.**, HENRIQUES, J. A., MARGIS, R., PASQUALI, G, REVERS, L.F.
Identification and expression analysis of genes associated with early berry development in the seedless grapevine (Vitis vinifera L.) cultivar Sultatine. Plant Science (Limerick). , v.179, p.510 - 519, 2010.

Capítulos de livros publicados

1. ROSA, S. B., CAVERZAN, A., RIBEIRO, C. W., LAZAROTTO, F., **PASSAIA, G.**, SILVEIRA, J. A., FERREIRA-SILVA, S. L., MARGIS, R., MARGIS-PINHEIRO, M.
Interação entre estresse oxidativo e estresses abióticos: o papel do sistema antioxidante na regulação das respostas de defesa. In: A FISIOLOGIA VEGETAL E OS DESAFIOS PARA PRODUÇÃO DE ALIMENTOS E BIOENERGIA. A FISIOLOGIA VEGETAL E OS DESAFIOS PARA PRODUÇÃO DE ALIMENTOS E BIOENERGIA.1 ed.Fortaleza : SPH Com Visual, 2009, v.1, p. 71-78.

Trabalhos publicados em anais de eventos (resumo)

1. **PASSAIA, G.**, CAVERZAN, A., FONINI, L., MARGIS-PINHEIRO, M.
FUNCTIONAL CHARACTERIZATION OF GLUTATHIONE PEROXIDASE GENE FAMILY IN RICE (ORYZA SATIVA). In: 10th International Conference on Reactive Oxigen and Nitrogen Species in Plants, 2011, Budapest.
10th International Conference on Reactive Oxigen and Nitrogen Species in Plants. , 2011.

2. **PASSAIA, G.**, CAVERZAN, A., FONINI, L., MARGIS-PINHEIRO, M.
FUNCTIONAL CHARACTERIZATION OF GLUTATHIONE PEROXIDASE GENE FAMILY IN RICE (ORYZA SATIVA). In: 57º Congresso Brasileiro de Genética, 2011, Aguas de Lindoia.
Livro de Resumos do 57º Congresso Brasileiro de Genética. , 2011.

3. **PASSAIA, G.**, CAVERZAN, A., FONINI, L., MARGIS-PINHEIRO, M.
Avaliação da expressão dos genes de glutationa peroxidase de arroz sob diferentes estresses abióticos e localização subcelular dos produtos gênicos In: XXVII REUNIÓN ARGENTINA DE FISIOLOGÍA VEGETAL,, 2010, La Plata.

LIBRO DE RESÚMENES Confrencias, Simposios y Trabajos. , 2010.

4. **PASSAIA, G.**, CAVERZAN, A., FONINI, L., MARGIS-PINHEIRO, M.
Functional Characterization of GLUTATHIONE PEROXIDASE GENE FAMILY IN RICE (ORYZA SATIVA). In: 8th International Symposium on Rice Functional Genomics, 2010, 2010, Bento Gonçalves/RS.
8th International Symposium on Rice Functional Genomics. , 2010.

5. **PASSAIA, G.**, REVERS, L.F., SBEGHEN, F., MARGIS-PINHEIRO, M.
Avaliação da expressão de genes regulados durante o desenvolvimento do fruto entre cultivares de uva de ciclo longo (Isabel e Cabernet Sauvignon) e ciclo curto (Isabel Precoce e Chardonay) In: XII Congresso Brasileiro de Viticultura e Enologia, 2008, Bento Gonçalves.
XII Congresso Brasileiro de Viticultura e Enologia. Bento Gonçalves: EMBRAPA UVA E VINHO, 2008.

6. **PASSAIA, G.**, SBEGHEN, F., MARGIS-PINHEIRO, M., REVERS, L.F.
Identificação de genes diferencialmente regulados durante o desenvolvimento do fruto das cultivares de uva Isabel e Isabel Precoce (Vitis labrusca) In: XI Congresso Brasileiro de Fisiologia Vegetal, 2007, Gramado-RS.
Brazilian Journal of Plant Physiology. , 2007.

7. SBEGHEN, F., **PASSAIA, G.**, REVERS, L.F.
Avaliação da resistência ao míldio numa população segregante do programa de melhoramento da EMBRAPA Uva e Vinho In: 52º Congresso Brasileiro de Genética, 2006, Foz do Iguaçú.
52º Congresso Brasileiro de Genética. Curitiba: São Franscisco Gráfica e Editora, 2006.

8. **PASSAIA, G.**, SBEGHEN, F., REVERS, L.F.
Caracterização do perfil de genes diferencialmente regulados durante o desenvolvimento de o fruto nas cultivares Isabel e Isabel Precoce In: 52º Congresso Brasileiro de Genética, 2006, Foz do Iguaçú.
52º Congresso Brasileiro de Genética. Curitiba: São Francisco Gráfica e Editora, 2006.