Universidade Federal do Rio Grande do Sul – UFRGS

Programa de Pós Graduação em Genética e Biologia Molecular

Inter-relações entre estresse oxidativo e estresses abióticos em arroz (*Oryza sativa* L.): o papel das ascorbato peroxidases nas respostas de defesa

Tese de Doutorado

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"*...Ao longo da jornada, vários obstáculos encontrei, muitas vezes me derrubaram, em todas elas, novamente levantei, não foi fácil, nem impossível de conquistar...*"

"Os fracos desistem e perdem, os fortes persistem, sofrem, lutam e vencem..."

SUMÁRIO

LISTA DE ABREVIATURAS

APX - ascorbato peroxidase

AsA - ascorbato

chlAPX - APX cloroplastídica

cAPX - APX citosólica

cm - centímetro(s)

cDNA - DNA complementar

CAT - catalase

cv - cultivar

CaMV - vírus do mosaico da couve flor, do inglês *"Cauliflower mosaic virus"*

dNTP - desoxirribonucleotídeos trifosfatados

dsRNA - RNA de fita dupla

DHA - dehidroascorbato

DHAR - dehidroascorbato redutase

DNA - ácido desoxirribonucleico

DAPI - 4´,6-diamino-2-fenilinodole

D.O. - densidade ótica

DTT - 1,4-ditiotreitol

ERO - Espécie Reativa de Oxigênio

EDTA - ácido etilenodiaminotetracético

g - grama (gramum - singular; grama - plural)

GFP - proteína fluorescente verde, do inglês "*Green Fluorescent Protein*"

GPX - glutationa peroxidase

GR - glutationa redutase

GSH - glutationa reduzida

GSSG - glutationa oxidada

h - hora(s)

kb - quilobase(s)

L - litro

LB - meio Luria-broth (meio de cultura universal para o cultivo de *Escherichia coli*)

M - molar

Mb - mega bases
mAPX - APX peroxissomal
min - minuto
mitAPX - APX mitocondrial
mRNA - RNA mensageiro
MS - meio de Murashige e Skoog (meio de cultura universal para o cultivo *in vitro* de tecidos vegetais)
ms - milissegundo
MDHA - monodehidroascorbato
MDHAR - monodehidroascorbato redutase
MV- metil viologênio
N- normal
$NADP^+$/NADPH - nicotinamida-adenina dinucleotídeo fosfato, oxidada/reduzida
NT - planta não-transformada
ng - nanograma
nM - nanomolar
nt- nucleotídeo
OsAPX - ascorbato peroxidase de *Oryza sativa*
pb - par (es) de bases
PCR - reação em cadeia da DNA-polimerase, do inglês "*Polymerase Chain Reaction*"
PEG - polietilenoglicol
PrxR - peroxirredoxina
PSI - fotossistema I
PSII- fotossistema II
RNA - ácido ribonucleico
RNAi - RNA de inteferência
rpm - rotação(ões) por minuto
s - segundo(s)
sAPX - APX estromal
SDS - dodecil sulfato de sódio
SOD - superóxido dismutase

T-DNA - DNA de transferência, sendo este parte do plasmídeo Ti de *Agrobacterium tumefaciens*

tAPX - APX tilacoidal

Tris - tri-hidroximetilaminometano

U - unidade de atividade enzimática

μg - micrograma

μL - microlitro(s)

μM - micromolar

μm - micrometro

μmol - micromol

μs - microssegundo

V - volume

x *g*- aceleração da gravidade

YFP - proteína fluorescente amarela, do inglês "*Yellow Fluorescent Protein*"

RESUMO

A ascorbato peroxidase (APX) é uma das principais enzimas do sistema de detoxificação de espécies reativas de oxigênio (ERO) em plantas, catalisando a conversão do peróxido de hidrogênio em água usando o ascorbato como doador de elétrons. No arroz, oito genes codificam APX. O presente estudo teve como objetivo avaliar o papel das enzimas de APXs em arroz, através da determinação das localizações subcelulares das diferentes isoformas, do estudo da expressão gênica e da caracterização detalhada do efeito do silenciamento das isoformas cloroplastídicas (chlAPXs) no metabolismo da planta, em condições normais e na resposta à estresses abióticos. A expressão diferencial das diferentes isoformas de APXs em condições de estresses abióticos varia entre as espécies e com a intensidade e duração do estresse. A localização subcelular de OsAPX1 e OsAPX2 no citosol, OsAPX3 e OsAPX4 nos peroxissomos, OsAPX5 no cloroplasto/mitocôndria, OsAPX6 na mitocôndria e OsAPX7 no cloroplasto foram confirmadas em protoplastos de arroz. Plantas duplamente silenciadas para os genes *OsAPX7/OsAPX8* não apresentam alterações fenotípicas, no entanto possuem alterações no metabolismo antioxidante sob condições normais de crescimento. Análises proteômicas dessas plantas revelaram que várias rotas importantes foram afetadas, especialmente proteínas envolvidas com processos fotossintéticos. Plantas silenciadas submetidas a estresse de alta luz (HL) e metil viologênio (MV) mostraram que as chlAPXs são importantes na proteção antioxidante em arroz. Alterações como fotodanos, fotoinibição e bloqueio do transporte de elétrons foram observadas nas plantas silenciadas submetidas a estresses abióticos, gerando grandes distúrbios na atividade fotoquímica. No entanto, sob alta luz as chlAPXs parecem não serem essenciais para a proteção de fotodanos, fotoinibição e danos oxidativos dirigidos pela alta luz no fotossistema II. Plantas silenciadas e não-transformadas (NT) exibem diferenças na fotossíntese sob condições normais de crescimento, bem como quando submetidas a estresses abióticos.

ABSTRACT

Ascorbate peroxidase (APX) is a major enzyme of the Reactive Oxygen Species (ROS) detoxification system in plants, catalyzing the conversion of hydrogen peroxide into water using ascorbate as electron donor. In rice eight genes encode APX. This study aimed to evaluate the role of APX enzymes in rice, through subcellular localization of the different isoforms, the study of gene expression and detailed characterization of chloroplastic APXs (chlAPXs) silencing in plant metabolism under normal conditions and in response to abiotic stresses. The differential expression of the different isoforms of APXs under abiotic stress conditions varies among species, and depends on the intensity and duration of stress. The subcellular localization of OsAPX1 and OsAPX2 in cytosol, OsAPX3 and OsAPX4 in peroxisomes, OsAPX5 in cloroplast/mitochondria, OsAPX6 in mitochondria and OsAPX7 in chloroplast were confirmed in rice protoplasts. Double silenced plants in *OsAPX7/OsAPX8* genes did not show phenotypic changes; however presented alterations in the antioxidant metabolism under normal growth conditions. Proteomic analysis revealed that in theses plants several important pathways were affected, especially proteins involved with photosynthetic process. Silenced plants subjected to high light (HL) and methyl viologen (MV) stresses show that the chlAPXs are important in the antioxidant protection in rice. Alterations such photodamage, photoinhibition and obstruction of the electron transport were observed in silenced plants subjected to abiotic stresses, generating great disturbances in photochemical activity. However, under high light the chlAPXs appear not be essential to the protection of photodamage, photoinhibition and oxidative damage triggered by HL in photosystem II (PSII). Silenced and non-transformed (NT) plants exhibit differences in photosynthesis under normal growth conditions, as well as, when subjected to abiotic stress.

1. INTRODUÇÃO GERAL

1.1 Espécies Reativas de Oxigênio

As Espécies Reativas de Oxigênio (ERO) são formadas a partir da redução univalente da molécula de oxigênio (O_2). Essas formas parcialmente reduzidas de oxigênio tais como o oxigênio singleto ($^1O_2$), o radical superóxido ($O_2^{\bullet-}$), peróxido de hidrogênio (H_2O_2) e o radical hidroxila (OH•) são ERO altamente reativas e tóxicas, podendo levar à destruição oxidativa das células (Mittler et al., 2004). As ERO são encontradas praticamente em todas as organelas intracelulares e nos diferentes compartimentos celulares como cloroplastos, mitocôndrias e peroxissomos devido à alta atividade metabólica normal desses compartimentos. Mehler em 1951 (revisão em Slesak et al., 2007) descreveu a fotorredução do O_2 nos cloroplastos *in vitro* e identificou o H_2O_2 como o produto final da reação. Posteriormente o $O_2^{\bullet-}$ foi identificado como o produto primário da fotorredução do O_2 nos tilacóides (Asada & Takahashi, 1987, revisão em Slesak et al., 2007). Atualmente a então chamada "Reação de Mehler" é considerada a mais poderosa fonte de H_2O_2/ERO no cloroplasto (Slesak et al., 2007). Nos peroxisomos as ERO são formadas na fotorrespiração, sendo a glicolato oxidase a principal via de geração de H_2O_2 (Dat et al., 2000) e nas mitocôndrias essas espécies reativas são geradas na cadeia transportadora de elétrons (Moller, 2001).

O H_2O_2 também é produzido no citoplasma, membrana plasmática e matriz extra celular. O citosol não é considerado grande fonte de ERO, porém atua como um depósito para estas moléculas derivadas de outros compartimentos subcelulares. O H_2O_2 gerado pelas NADPH oxidases, amino oxidases e peroxidases da parede celular são altamente reguladas e geralmente participam da produção de ERO durante processos como morte celular programada e defesa a patógenos (Dat et al., 2000; Grant & Loake, 2000; Mittler 2002; Mittler et al., 2004).

Cada organela ou compartimento celular têm potenciais alvos para danos oxidativos, bem como mecanismos para eliminação do excesso de acumulação de ERO. O equilíbrio entre a produção e eliminação de ERO pode ser perturbado

por vários fatores de estresses bióticos e abióticos (Mittler, 2002; Scandalios, 2002). Esses distúrbios no equilíbrio conduzem a um rápido aumento no nível intracelular de ERO o qual pode causar significantes danos às estruturas celulares (Gill & Tuteja, 2010). Bhattachrjee (2005) estimou que 1 a 2% do consumo de O_2 é desviado para a formação de ERO em tecidos vegetais. O H_2O_2 é considerado moderadamente reativo, possui uma meia-vida relativamente longa (1 ms), enquanto que outras ERO como o $O_2^{\bullet-}$, OH• e $^1O_2$ possuem uma meia-vida mais curta (2 - 4 µs) (Bhattachrjee, 2005). Essas moléculas afetam muitas funções celulares, danificando ácidos nucléicos, lipídios e proteínas, resultando até mesmo na morte celular (Scandalios, 2005).

As plantas possuem mecanismos para neutralizar a acumulação de ERO induzida por estresse. Esses mecanismos de detoxificação incluem os sistemas antioxidantes enzimáticos e os não-enzimáticos. O sistema de detoxificação não-enzimático inclui o ascorbato (AsA), glutationa (GSH), tocoferol, carotenoides, flavonoides e componentes fenólicos (Mittler et al., 2004; Gratão et al., 2005; Scandalios, 2005). O ascorbato e a glutationa são os principais antioxidantes não-enzimáticos em plantas atuando na defesa contra o estresse oxidativo, exercendo o papel de tampões antioxidantes. Ambos, AsA e GSH são abundantes e estáveis antioxidantes com apropriado potencial redox que interagem com numerosos componentes e vias. Essas moléculas são mantidas geralmente no seu estado reduzido por um conjunto de enzimas dependente de NAD(P)H tais como: monodehidroascorbato redutase, dehidroascorbato redutase e glutationa redutase (Mittler et al., 2004; Foyer & Noctor, 2005, 2011).

O mecanismo de detoxificação enzimático nas células vegetais inclui várias enzimas que atuam na eliminação de ERO tais como a superóxido dismutase (SOD, EC 1.15.1.1), ascorbato peroxidase (APX, EC 1.11.1.11), glutationa peroxidase (GPX, EC 1.11.1.9), catalase (CAT, EC 1.11.1.6) e peroxiredoxina (PrxR) (Mittler et al., 2004). O balanço entre SOD, CAT e APX é crucial para determinar o nível intracelular efetivo de $O_2^{\bullet-}$ e o H_2O_2. A principal via de detoxificação de ERO em plantas incluem o ciclo do ascorbato-glutationa nos cloroplastos, mitocôndrias, peroxissomos, citosol e apoplasto e a catalase nos peroxissomos (Mittler et al., 2004; Teixeira et al., 2004).

A redução do oxigênio molecular a superóxido na “Reação de Mehler” no cloroplasto é o primeiro passo de uma série de reações que juntas têm sido chamadas de ciclo “Water-Water” (Asada, 1999). A enzima superóxido dismutase atua na linha de frente na defesa contra ERO convertendo o $O_2^{\bullet-}$ produzido na “Reação de Mehler” a H_2O_2 (Figura 1). Posteriormente, CAT, APX e GPX detoxificam H_2O_2. O ciclo do ascorbato-glutationa, também referido como “Foyer-Halliwell-Asada” (Foyer & Halliwell, 1976, 1977; Asada, 1999; Foyer & Noctor, 2011) é a via mais importante de eliminação de ERO em plantas.

Devido sua atuação em quase todos os compartimentos celulares e a alta afinidade da APX por H_2O_2, sugere-se que o ciclo do ascorbato-glutationa desempenha uma função crítica controlando os níveis de ERO nos diferentes compartimentos celulares (Mittler, 2002). Ele envolve sucessivas oxidações e reduções do ascorbato, glutationa e NADPH pelas enzimas ascorbato peroxidase, glutationa redutase (GR, EC 1.6.4.2), dehidroascorbato redutase (DHAR, EC 1.8.5.1) e monodehidroascorbato redutase (MDHAR, EC 1.6.5.4) (Teixeira et al., 2004; Gratão et al., 2005). O radical monodehidroascorbato, gerado pela oxidação do ascorbato pela APX durante a detoxificação do peróxido de hidrogênio é regenerado por duas vias: ele pode ser convertido à ascorbato pela ação da enzima monodehidroascorbato redutase utilizando NAD(P)H ou, então, devido à sua instabilidade, ele pode gerar o radical dehidroascorbato em uma reação não enzimática. Em seguida, o dehidroascorbato é regenerado a ascorbato pela enzima dehidroascorbato redutase utilizando a glutationa como fonte redutora. Por fim, a glutationa oxidada é recuperada pela ação da glutationa redutase utilizando NAD(P)H (Figura 1) (Teixeira et al., 2004).

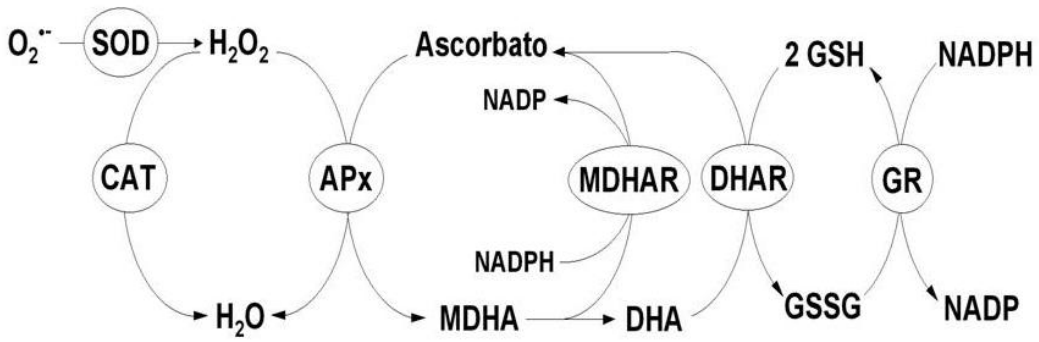

Figura 1. Via de eliminação de ERO em plantas. APX – Ascorbato peroxidase; **CAT** – Catalase; **SOD** – Superóxido dismutase; **DHA** – Dehidroascorbato; **DHAR** – Dehidroascorbato redutase; **MDHA** – Monodehidroascorbato; **MDHAR** – Monodehidroascorbato-redutase; **GR** – Glutationa redutase; **GSH** – Glutationa reduzida; **GSSG** – Glutationa oxidada; (Teixeira et al., 2004).

A enzima catalase presente nos peroxissomos também é indispensável para detoxificação de ERO durante o estresse oxidativo, no entanto, ao contrário da APX, a CAT não requer suprimentos de moléculas doadoras de elétrons. Além disso, APX e CAT possuem diferenças de afinidade pelo H_2O_2, o que sugere funções diferentes entre as duas enzimas. A APX (em nível de µM H_2O_2) parece estar envolvida na fina modulação de ERO, enquanto a CAT (em nível de mM H_2O_2) deve atuar na remoção de ERO durante estresse (Mittler, 2002).

Recentemente muitos estudos têm demonstrado que as ERO controlam diferentes processos fisiológicos em plantas. A função das ERO como moléculas sinalizadoras envolvidas em processos como crescimento, ciclo celular, desenvolvimento, senescência, morte celular programada, condutância estomatal, sinalização hormonal, regulação da expressão gênica e reposta a estresses abióticos e bióticos têm sido amplamente explorada (Kovtun et al., 2000; Neill et al., 2002; Slesak et al., 2007; Inze et al., 2012). O uso das ERO como moléculas sinalizadoras pelas células vegetais sugere que ao longo da evolução as plantas foram capazes de alcançar um elevado grau de controle sobre a toxicidade de ERO e agora usam as mesmas como moléculas sinalizadoras (Mittler et al., 2004). O duplo papel das ERO no metabolismo celular, atuando como produtos tóxicos e como reguladoras de processos celulares e vias de defesas, é dependente da sua concentração intracelular (Foyer & Noctor, 2005).

Entre as várias ERO, o H_2O_2 é considerado a principal molécula envolvida na sinalização. Muitos estudos relatam o aumento na concentração de H_2O_2 após exposição a um estresse, no entanto a taxa de produção do H_2O_2 depende da intensidade e duração do estresse. Além disso, o nível de H_2O_2 difere entre os vários compartimentos celulares e está relacionado ao tipo de estresse (Slesak et al., 2007).

O H_2O_2 pode se difundir cruzando as membranas celulares e pode ser transportado para outros compartimentos onde pode atuar como uma molécula sinalizadora ou ser removido. A difusão do H_2O_2 pelas membranas é facilitada pelas proteínas aquaporinas que atuam como canais de membrana peroxiporinas, e deste modo facilitam a difusão do H_2O_2 que cruza as membranas biológicas (Neill et al., 2002; Hooijmaijers et al., 2012). Apesar da importância das ERO como moléculas sinalizadoras, sérios problemas persistem na capacidade de quantificar o H_2O_2 e outras formas de ERO e também em distinguir as mesmas nos diferentes eventos de sinalização (Foyer & Noctor, 2012). Além disso, como esses sinais são percebidos, transmitidos e como eles provocam uma resposta específica em diferentes espécies de plantas não foi ainda bem esclarecido (Gadjev et al., 2006).

1.2 Estresse Oxidativo, Biótico e Abiótico e as Defesas Antioxidantes

O crescimento e a produtividade das culturas são negativamente afetados por vários fatores abióticos e bióticos. No campo, as plantas são frequentemente expostas a uma ampla variedade de condições de estresses, tais como baixas e altas temperaturas, radiação UV, salinidade, seca, inundação, herbicidas, ataque de patógenos, estresse oxidativo e toxicidade por metais pesados. Acredita-se que várias atividades antropogênicas tenham acentuado os fatores de estresses existentes (Mahajan & Tuteja, 2005; Gill &Tuteja, 2010). As rotas metabólicas nas organelas das plantas são sensíveis a mudanças nas condições ambientais (Suzuki et al., 2012). Dessa forma, vários estresses ambientais impostos nos tecidos vegetais induzem mudanças no metabolismo do oxigênio que causa estresse oxidativo.

O estresse oxidativo ocorre quando há um aumento na produção de ERO em algum compartimento celular, e essas não são rapidamente eliminadas (Mullineaux & Baker, 2010). Além disso, juntamente ocorre um sério desequilíbrio nas defesas antioxidantes que dificulta as atividades metabólicas (Gratão et al., 2005). Desse modo, ocorrem danos oxidativos aos componentes celulares que podem levar à perda da função e eventual morte celular (Mullineaux & Baker,

2010). Foyer & Shigeoka (2011) relataram que o H_2O_2 é um potente inibidor de muitos processos incluindo a fotossíntese, pois mesmo em baixas concentrações (10 µM) ele pode inibir a fixação de CO_2 a 50%, devido à oxidação das enzimas moduladas pelo grupo tiol do ciclo de Calvin. Desse modo o balanço entre a produção e eliminação de ERO nos cloroplastos é delicado e deve ser rigorosamente controlado.

Muitos estudos demonstram a complexidade dos mecanismos de produção e detoxificação de ERO, bem como o efeito do estresse oxidativo no sistema antioxidante em diferentes espécies de plantas. Em *Arabidopsis*, foi observado o envolvimento de pelo menos 152 genes na regulação do nível de ERO sob estresses (Mittler et al., 2004). O estresse com baixa temperatura (8 ^{o}C) durante quatro dias em plantas de pepino aumentou significantemente a taxa de produção de $O_2^{\bullet-}$ e também o conteúdo de H_2O_2, além de fortemente aumentar a atividade da SOD e APX (HU et al., 2008). Raízes de plantas arroz submetidas ao tratamento com cloreto de cádmio aumentaram a produção de H_2O_2, além disso, o estresse inibiu a atividade da enzima CAT, porém não teve nenhum efeito na atividade da SOD, APX e GR (Cho et al., 2012). Plantas de tabaco com redução da atividade APX induziram CAT, SOD e GR como um mecanismo de compensação da deficiência da APX. Já plantas com supressão da enzima CAT induziram APX, enquanto que plantas deficientes para ambas as enzimas CAT e APX não induziram genes de resposta a estresses. Além disso, essas plantas foram mais tolerantes a estresses oxidativos que as plantas com redução de apenas APX ou CAT (Rizhsky et al., 2002).

A superexpressão de duas enzimas antioxidantes, SOD e APX em tabaco aumentou a longevidade da semente e a taxa de germinação em condições de estresses abióticos. As sementes superexpressando SOD e APX mostraram um aumento significante na taxa de germinação em todas as combinações de estresse testadas (osmótico/sal, baixa temperatura/osmótico, baixa temperatura/sal e baixa temperatura/osmótico/sal), demonstrando a importância dessas enzimas no aumento da tolerância a estresses abióticos (Lee et al., 2010). Por outro lado, plantas de *Arabidopsis* mutantes deficientes em APX acumularam mais H_2O_2 e foram mais sensíveis ao estresse combinado de seca e calor do que

plantas selvagens (Koussevitzky et al., 2008). As evidências experimentais descritas acima confirmam a importância das enzimas antioxidantes e a complexidade do metabolismo das ERO nos vegetais.

1.3 Ascorbato peroxidase

No presente trabalho abordamos os aspectos moleculares, bioquímicos e fisiológicos da enzima ascorbato peroxidase, uma das principais enzimas envolvidas no sistema antioxidante vegetal.

As peroxidases atuam nas defesas antioxidantes removendo os peróxidos, incluindo o H_2O_2, dessa forma desempenham um importante papel no sistema antioxidante controlando as ERO. Amplamente distribuída em plantas, a enzima ascorbato peroxidase é uma heme-peroxidase de classe I e como já mencionado, utiliza o ascorbato como doador de elétrons específico para reduzir H_2O_2 à água. Dessa forma a APX é uma das enzimas mais importantes atuando no ciclo do ascorbato-glutationa, na detoxificação dos níveis tóxicos de H_2O_2 (Asada, 1992, 1999; Shigeoka et al., 2002). As APXs possuem um grupamento heme (protoporfirina IX) como grupo prostético e são inibidas por alguns reagentes como azida, cianida, hidroxilamina, hidroxiureia e *p*-amino-phenol. Tais reagentes são utilizados em ensaios para distinguir a APX de outras peroxidases (Amako et al., 1994; Chen & Asada, 1989; Shigeoka et al., 1980, 2002). Uma das propriedades características das APXs é sua instabilidade na ausência de ascorbato (Chen & Asada, 1989; Shigeoka et al., 2002; Ishikawa & Shigeoka, 2008).

Muitos estudos têm sido realizados abrangendo aspectos bioquímicos, moleculares e fisiológicos da enzima APX e a sua relação com outros componentes do sistema antioxidante enzimático e não enzimático. Esses dados juntamente com os dados de expressão dos genes de *APX* frente a uma variedade de estresses demonstram a importância da enzima APX em organismos fotossintéticos. Em vários desses estudos foi observado que a atividade da APX geralmente aumenta simultaneamente com a atividade de outras enzimas antioxidantes como CAT, GSH e SOD em resposta a vários

fatores de estresses ambientais, sugerindo uma co-regulação entre os componentes do sistema antioxidante (Shigeoka et al., 2002; Mittler et al., 2004).

Em arroz, foi identificada uma família multigênica de ascorbato peroxidase formada por oito genes (Teixeira et al., 2004; Teixeira et al., 2006). O nosso grupo vem desenvolvendo pesquisas relacionadas com a análise evolutiva e funcional dos genes que codificam essas enzimas (Menezes-Benavente et al., 2004, Teixeira et al., 2004, Teixeira et al., 2005, Teixeira et al., 2006, Rosa et al., 2010; Lazzarotto et al., 2011, Bonifacio et al., 2011, Ribeiro et al., 2012). Além disso, plantas transgênicas de arroz silenciadas nos genes das isoformas citosólicas e peroxissomais de APX foram produzidas e as análises fisiológicas destas plantas mostraram que esses genes além de envolvidos nas respostas a estresse, participam também de processos relacionados com o desenvolvimento da planta (Rosa et al., 2010; Bonifacio et al., 2011; Lazzarotto et al., 2011).

O papel das isoformas de APX de arroz localizadas nos cloroplastos não foi ainda avaliado. A hipótese a ser investigada nesta tese é de que as APX cloroplastídicas, diferentemente das APX citosólicas, estão mais relacionadas com as respostas a estímulos ambientais que envolvam os fotossistemas do que com os processos ligados ao desenvolvimento. Para testar essa hipótese, em primeiro lugar é necessário confirmar a localização das isoformas nos diferentes compartimentos subcelulares e avaliar os efeitos das modificações na expressão desses genes em plantas geneticamente modificadas.

1.4 A espécie *Oryza sativa* L.

O arroz (*Oryza sativa* L.) pertence à divisão angiosperma, à classe das monocotiledôneas, à família Poaceae. A espécie *Oryza sativa* L. é a mais conhecida por sua importância na alimentação humana, sendo a espécie mais cultivada (Gomes & Magalhães Júnior, 2004). O arroz é considerado a planta modelo, entre as monocotiledôneas, para estudos biológicos. Este possui um genoma relativamente pequeno (390 Mb) quando comparado ao genoma de outros cereais como o milho, cevada e trigo que têm seus genomas estimados em

3000, 5000 e 16000 Mb, respectivamente. Uma versão do genoma do arroz sequenciado foi publicada pelo International Rice Genome Sequence Project (IRGSP, 2005). Posteriormente, em 2007, uma anotação acurada do genoma de *Oryza sativa* L. *ssp*. *japonica* foi publicada, sugerindo que o número de genes em arroz é aproximadamente 32.000 genes (The Rice Annotation Project, 2007).

Além da disponibilidade do genoma completo, foram desenvolvidas várias ferramentas que reforçam o papel do arroz como uma planta modelo: i) mapa genético saturado (International Rice Genome Sequencing Project, http://rgp.dna.affrc.go.jp/IRGSP/) (Harushima et al., 1998); ii) técnica eficiente de transformação genética (Hiei et al., 1994); mapas genéticos comparativos entre os cereais (Moore et al., 1995). Estes últimos revelaram a existência de regiões extensas de conteúdo gênico conservado e ordenado entre os cereais, ou seja, a existência de intensa sintenia entre as gramíneas (Moore et al., 1995). O arroz juntamente com o trigo e o milho são os cereais mais cultivados do mundo, suprindo cerca de 50% das calorias consumidas pela população mundial (Vij et al., 2006).

Cultivado e consumido em todos os continentes, o arroz destaca-se pela produção e área de cultivo, contribuindo tanto no aspecto econômico quanto social. Aproximadamente 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. 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 10,6 milhões de toneladas. A cultura do arroz ocupa a sétima posição no Brasil entre as culturas mais plantadas (http://www.faostat.fao.org). A produção do cereal é oriunda do sistema de cultivo de várzea e de terras altas, sendo o estado do Rio Grande do Sul o maior produtor com aproximadamente 68% da produção nacional (Gomes & Magalhães Júnior, 2004).

No Brasil, a produtividade do arroz, assim como para outras culturas, depende da capacidade destas de responder a diferentes tipos de adversidades ambientais, os quais geralmente produzem um estresse oxidativo (Gomes & Magalhães Júnior, 2004). A caracterização funcional de genes que possam proteger as plantas contra esses danos oxidativos pode contribuir para o desenvolvimento de plantas mais adaptadas ao meio ambiente.

2. OBJETIVOS

O objetivo geral deste trabalho foi avaliar o papel das enzimas de ascorbato peroxidase em arroz, através da determinação das localizações subcelulares das diferentes isoformas, do estudo da expressão gênica e da caracterização detalhada do efeito do silenciamento das isoformas cloroplastídicas no metabolismo da planta em condições normais e na resposta a estresses abióticos.

2.1. Objetivos específicos

2.1.1. Determinar experimentalmente a localização subcelular das proteínas APX em arroz.

2.1.2. Obter e caracterizar plantas silenciadas para os dois genes *OsAPX7* e *OsAPX8* simultaneamente (isoformas cloroplastídicas de APX) via RNA de interferência;

2.1.3. Analisar fenotipicamente as plantas silenciadas e não-transformadas (NT) crescidas em condições normais e submetidas a estresses abióticos;

2.1.4. Avaliar o padrão de expressão da família gênica de *OsAPX* nas plantas silenciadas em condições normais e submetidas a estresses abióticos;

2.1.5. Analisar fisiológica e bioquimicamente as plantas silenciadas e não-transformadas crescidas em condições normais e submetidas a estresses abióticos;

2.1.6. Identificar as alterações produzidas pelo silenciamento no conjunto de proteínas expressas através de análise proteômica das plantas silenciadas crescidas em condições normais;

Visando atender os objetivos acima descritos, essa tese está organizada em três capítulos. No capítulo 1 é apresentada uma revisão sobre a regulação da expressão dos genes de *APX* em diversas espécies de plantas em condições normais e submetidas a estresses visando descrever o estado da arte em relação

ao papel de proteção dessas enzimas contra os estresses ambientais. O capítulo 2 contempla o objetivo específico 1 desta tese onde são apresentados os dados referentes à localização subcelular das proteínas de APX em protoplastos de arroz. No capítulo 3 são apresentados os resultados relativos ao estudo funcional das isoformas cloroplastídicas de ascorbato peroxidase em arroz (*Oryza sativa* L.), abordando aspectos moleculares, fisiológicos e bioquímicos das respostas ao silenciamento dessas enzimas em uma planta monocotiledônea.

CAPÍTULO 1

Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection

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Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection

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ABSTRACT

When plants are exposed to stressful environmental conditions, the production of Reactive Oxygen Species (ROS) increases and can cause significant damage to the cells. Antioxidant defenses, which can detoxify ROS, are present in plants. A major hydrogen peroxide - a ROS - detoxifying system in plant cells is the ascorbate-glutathione cycle, in which, ascorbate peroxidase (APX) enzymes play a key role catalyzing the conversion of H_2O_2 into H_2O, using ascorbate as a specific electron donor. Different APX isoforms are present in distinct subcellular compartments, such as chloroplasts, mitochondria, peroxisome, cytosol, and nucleus. The *APX* genes expression is regulated in response to biotic and abiotic stresses as well as during plant development. The APX responses are directly involved in the protection of plant cells against adverse environmental conditions. Besides, mutant plants *APX* genes showed alterations in growth, physiology and antioxidant metabolism revealing those enzymes involvement in the normal plant development.

Keyword: ascorbate peroxidase - antioxidant system - reactive oxygen species - abiotic stress - mutant plants

INTRODUCTION

The exposure of plants to unfavorable environmental conditions increases the production of reactive oxygen species (ROS) such as, singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet}$). The ROS detoxification process in plants is essential for the protection of plant cells and their organelles against the toxic effect of these species (Mittler, 2002; Apel and Hirt, 2004). The differences in subcellular localization and biochemical properties of antioxidant enzymes and the distinct responses in gene expression, in addition to the presence of non-enzymatic mechanisms, result in a versatile and flexible antioxidant system able to control the optimum ROS levels (Vranova et al., 2002). The ROS detoxification systems include enzymatic and non-enzymatic antioxidant components (Scandalios, 2005). Ascorbate (AsA) and glutathione (GSH), non-enzymatic antioxidants are crucial for plant defense against oxidative stress, playing a key role as antioxidant buffers (Mittler, 2002; Foyer and Noctor, 2005). Other non-enzymatic antioxidants involved include flavonoids, phenolic compounds, alkaloids, tocopherol and carotenoids (Gratão et al., 2005).

Enzymatic antioxidants comprise superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR). These enzymes are present in practically all subcellular compartments. Usually, an organelle has more than one enzyme able to scavenge a single ROS (Mittler, 2002; Mittler et al., 2004; Scandalios, 2005). The main hydrogen peroxide-detoxification system in plant chloroplasts is the ascorbate-glutathione cycle, in which APX is a key enzyme (Asada, 1992). APX utilizes AsA as specific electron donor to reduce H_2O_2 to water. The importance of APX and ascorbate-glutathione cycle is not restricted to chloroplasts; it also plays a role in ROS scavenging in cytosol, mitochondria and peroxisomes (Asada, 1992; Noctor and Foyer, 1998; Asada, 1999; Shigeoka et al., 2002; Mittler et al., 2004). The

ROS-scavenging enzymes in plants has been widely studied and the results have demonstrated that APX activity generally increases along with other enzymes activities, such as CAT, SOD, and GSH reductase, in response to environmental stress (Shigeoka et al., 2002). Over the past ten years the effort to understand the plant antioxidant system mechanisms has been intensified (Fig.1). The increasing number of publications addressing CAT, SOD, GPX and APX enzymes in plants are examples of this tendency, especially APX, which had the number of articles doubled from 158 published in 2000 to 368 in 2010 (ISI Web of Knowledge database). Publications related more specifically to ROS in plants increased 18 times, considering the same period. The data presented in this study confirm those related by Azevedo and Azevedo (2006) in which the number of publications addressing antioxidant mechanisms increased after 2000 (Azevedo and Azevedo, 2006). This shows the relevance of studying those enzymes to further understand the biological processes dealing with oxidative stress responses in plants. The focus of this review is the discussion of the main findings related to the APX enzyme at molecular and physiological levels, in different plant species. The *APX* gene modulation in response to abiotic stress conditions; especially temperature, high light, drought, salinity and heavy metals will be reviewed.

Ascorbate Peroxidase in Plants

Ascorbate peroxidase (APX) (EC 1.11.1.11) belongs to the class I heme-peroxidases and it is found in higher plants, chlorophytes (Takeda et al., 1998; Takeda et al., 2000), red algae (Sano et al., 2001), and members of the protist kingdom (Shigeoka et al., 1980; Wilkinson et al., 2002). APX and other peroxidase sequences from all kingdoms of life are stored in the database Peroxibase (Oliva et al., 2009). This web site (http://peroxibase.isb-sib.ch) also provides a series of bioinformatics tools and facilities useful for analyzing the peroxidases stored sequences. Genomic and cDNA *APX* sequences were obtained from a great variety of plant species, showing that APX are widely distributed in the vegetal kingdom. These enzymes are encoded by small gene families in these organisms (Passardi et al., 2007). The different isoforms are classified according to their

subcellular localization. Soluble isoforms are found in cytosol (cAPX), mitochondria (miAPX) and chloroplast stroma (sAPX), while membrane-bound isoforms are found in microbody (including peroxisome and glyoxisome) (mAPX) and chloroplast thylakoids (tAPX). Recently, we have detected cytosolic APX1 and APX2 in the nucleus of protoplasts isolated from rice leaves (unpublished results). The presence of organelle-specific targeting peptides and transmembrane domains found in the N- and C- terminal protein regions determine the final subcellular localization of the isoenzyme (Shigeoka et al., 2002; Teixeira et al., 2004; Teixeira et al., 2006).

Plant chloroplastic APX (chlAPX) isoenzymes encoding genes are divided into two groups. The first group comprises single genes encoding two isoenzymes through a post-transcriptional alternative splicing regulation. This group includes genes from spinach (*S. oleracea*), tobacco (*N. tabacum*), pumpkin (*Cucurbita sp*) and ice plant (*M. crystallium*). In the second group, individual genes codify different isoenzymes, which are individually regulated. This group includes genes from *Arabidopsis,* rice, and tomato. The mechanism of alternative splicing in chlAPX has been studied in spinach (Ishikawa and Shigeoka, 2008) and the results showed that alternative splicing is fundamental for controlling the expression of stromal (sAPX) and thylakoid (tAPX) isoenzymes. This regulation occurs in a tissue-dependent manner.

Ascorbate peroxidases have been partially characterized in some plant species. In spinach, APX family is formed by genes encoding one cytosolic, two chloroplastic (sAPX and tAPX membrane) isoenzymes, one targeted to microbody membrane and a unknown putative cytosol-soluble isoenzyme (Ishikawa et al., 1995; Ishikawa et al., 1996; Ishikawa et al., 1998). In cowpea, four cDNAs were isolated and characterized, corresponding to putative cytosolic, peroxisomal and chloroplastic (thylakoid and stromal) APX isoforms (D'Arcy-Lameta et al., 2006). Six loci encoding *APX* were identified in *Eucalyptus grandis* and their subcellular localizations were indicated by prediction programs. Among the six isoforms, three were putatively identified as cytosolics, one as a putative peroxisomal protein and two predicted to be associated with chloroplasts (Teixeira et al., 2005). In tomato,

seven members were identified, three cytosolics, two peroxisomals, and two chloroplastics (Najami et al., 2008). In the model plant *Arabidopsis thaliana*, it was described the presence of nine *APX* genes, two choroplastics, one thylakoid-bound and one member whose product is targeted to both chloroplast stroma and mitochondria (Chew et al., 2003), however the intracellular localization of an additional member is unknown. In addition, three cytosolics and three microsomals proteins were also described (Panchuk et al., 2002; Mittler et al., 2004; Narendra et al., 2006). In another important model plant, rice, the *APX* gene family comprises eight members: two cytosolics, two peroxisomals, two chloroplastics (stromal and thylakoid-bound), and two mitochondrials (Teixeira et al., 2004; Teixeira et al., 2006). Recently, a new protein has also been identified functionally associated with APX in rice, the APX-R (Ascorbate peroxidase-related) (Lazzarotto et al., 2011). Detailed analyses of evolution and structure of *APX-R* genes indicate that these genes correspond to a new class of heme-peroxidases (Lazzarotto et al., 2011).

APX isoenzymes are labile in the absence of AsA. Thus, high level of endogenous AsA is essential to effectively maintain the antioxidant system that protects plants from oxidative damage (Asada, 1992; Shigeoka et al., 2002). Under special conditions in which the concentration of AsA is lower than 20 µM, the APX activity is quickly lost what makes the chlAPX the most unstable isoform. The cAPX and mAPX have half-inactivation time around of one hour or more while that miAPX and chlAPX is less than 30 s (Chen and Asada, 1989; Miyake et al., 1993; Ishikawa et al., 1998; Yoshimura et al., 1998; Leonardis et al., 2000).

APX enzymes response under abiotic stress

The expression of *APX* encoding genes is modulated by various environmental stimuli, such as drought and salt stress, high light, high and low temperatures, pathogen attacks, H_2O_2 and abscisic acid (Zhang et al., 1997; Yoshimura et al., 2000; Agrawal et al., 2003; Fryer et al., 2003; Menezes-Benavente et al., 2004; Teixeira et al., 2006; Rosa et al., 2010; Bonifacio et al.,

2011). Besides, the transcriptional expression of *APX* genes is tissue and developmental stage dependent (Agrawal et al., 2003; Teixeira et al., 2006).

Salt stress

Plants are greatly affected by salinity, which causes alteration in nutrient uptake, accumulation of toxic ions, osmotic stress, and oxidative stress (Verslues et al., 2006). Consequently, salinity results in molecular damage, growth arrest, and even cell death (Wang et al., 2008). Salt stress induces the production of ROS, and the response of *APX* genes to this condition is tissue and developmental stage regulated. When the response of major antioxidant enzymes transcripts, under salt stressed rice in different developmental stages was analyzed, *cAPX* was up-regulated in 11-day-old seedlings, while in 6-week-old plants salt had no significant effect on this enzyme (Menezes-Benavente et al., 2004). In addition to *APX* expression alteration, discrimination on *CAT* transcription accumulation was also noticed in the basal region of rice leaves under salinity (Yamane et al., 2010). Concerning the APX rice isoforms, induction was observed in *OsAPX1*, *OsAPX4*, *OsAPX6* and *OsAPX7* genes, whereas the cytosolic *OsAPX2* gene expression was not altered by salinity (Yamane et al., 2010).

Teixeira et al. (2006) reported that three rice *APX* genes (*OsAPX2*, *OsAPX7*, and *OsAPX8*) showed altered transcript levels in response to NaCl treatment. The expression of *OsAPX2* and *OsAPX7* was increased, whereas the *OsAPX8* transcripts accumulation was strongly suppressed in plants subjected to salt stress (Teixeira et al., 2006). The transcript level of *OsAPX8* was slightly decreased by salinity in the basal region of rice leaves (Yamane et al., 2010). On the other hand, *OsAPX8* expression in rice roots was enhanced by all NaCl tested concentrations (150-300 mM) and *OsAPX7* expression was down-regulated by 300 mM NaCl. This discrepancy in regulation of the *OsAPX* genes might be due to differences in cultivars, organs, plant age and growth conditions (Hong et al., 2007). Increment of rice cytosolic *APX2* gene transcript after treatment with salt

has previously been shown by our group (Menezes-Benavente et al., 2004; Teixeira et al., 2006).

In accordance, transgenic plants over-expressing cytosolic *OsAPXb* (*OsAPX2*) showed higher tolerance to NaCl than those over-expressing cytosolic *OsAPXa* (*OsAPX1*) in *Arabidopsis* (Lu et al., 2007). A similar increment in salt stress tolerance was also observed in transgenic tobacco over-expressing the *Arabidopsis cAPX* gene (Badawi et al., 2004) and also in tobacco plants over-expressing *Solanum lycopersicum* thylakoid-bound ascorbate peroxidase gene (*StAPX*) (Sun et al., 2010). Transgenic tobacco plants that simultaneously express CuZnSOD, APX, and DHAR in chloroplasts presented increased protection against salt induced injury (Lee et al., 2007). Transgenic tobacco BY-2 cells with 50 and 75% lower cAPX activity showed higher intracellular content of ROS. On the other hand, the tobacco cells showed a potential enhancement in tolerance to heat and salt stress, perhaps by induction of stress-related gene expression. However, no substantial differences in the activity levels of the other antioxidant enzymes were observed (Ishikawa et al., 2005).

In barley, the transcript level of peroxisomal *APX* gene (*HvAPX1*) increased significantly under salt stress (Shi et al., 2001). However, *Arabidopsis apx3* knockout mutants exposed to stressful conditions did not present disturbed growth or development. In these plants, other antioxidant enzymes possibly compensate the lack of peroxisomal isoform (Narendra et al., 2006). Whereas, the overexpression of Populus peroxisomal *APX* (*PpAPX*) gene in transgenic tobacco improved salt tolerance at the vegetative stage and plants were more resistant to oxidative damage induced by methyl viologen (MV). In addition, the plants had higher root length (Li et al., 2009).

Lin and Pu (2010) studied changes in enzymes involved in ROS scavenging in tolerant and sensitive to salinity sweet potato plants. APX activity increased in plants after exposure to salinity (450 mM NaCl) for 24 and 48 h in higher extension in salt-stress tolerant genotype than in the salt sensitive ones. The expression of *cAPX*, *mAPX* and *chlAPX* in response to salinity was tissue specific and dependent on stress duration (Lin and Pu, 2010). Taken together, these studies

have demonstrated that salt stress caused disturbances in antioxidant gene expression, producing alterations in the transcriptional pattern in several plant species, and that distinct APX isoform expressions may result in the redox homeostasis regulation in each cellular compartment.

Temperature stress

Extreme temperature affects the growth, yield and quality of plant production. ROS levels tend to increase if plants are exposed to stressful conditions such as low or high temperatures (Mittler et al., 2004; Scandalios, 2005). The transient accumulation of *cAPX* mRNA after low-temperature storage was greater than after high-temperature storage in potato tubers showing that *APX* expression was induced in response to low temperature (Kawakami et al., 2002). Likewise, the two rice *cAPX* (*OsAPX1* and *OsAPX2*) genes were induced after rice plants exposure to low temperature. Besides, *OsAPX3*, *OsAPX4, OsAPX6* and *OsAPX7* were also significantly induced, while *OsAPX8* were repressed after 24 h under low temperature (unpublished data). The sweet potato *cAPX* gene was highly induced in leaves after plant exposure to high temperature (Park et al., 2004).

In cucumber plants subjected to heat treatments, the activities of cAPX, sAPX, and mAPX, increased after an initial and small decline during the course of the experiment. The expression of *sAPX* followed a similar change pattern (Song et al., 2005). In response to cold, the expression of a peroxisomal *APX* gene increased slightly in *Arabidopsis* (Zhang et al., 1997). Corroborating to this study, when a putative peroxisomal membrane-bound *APX* from barley was overexpressed in *Arabidopsis*, an increased tolerance to higher temperature treatment was observed (Shi et al., 2001). Besides, the overexpression of chloroplastic *tAPX* in tobacco plants improved the tolerance to chilling stress with high light intensity (Yabuta et al., 2002). On the other hand, *Arabidopsis* plants lacking *tAPX* had enhanced tolerance to heat stress (Miller et al., 2007). Recently, Sato et al. (2011) have showed that transgenic rice plants overexpressing

cytosolic *APX1* gene (*OsAPXa*), which exhibited APX activities in spikelets higher than in wild type (WT) plants, sustained higher levels of APX activity under cold stress, resulting in enhanced cold tolerance at the booting stage in rice.

Plants with enhanced tolerance to multiple environmental stresses were obtained through the expression of *CuZnSod* and *APX* genes. *Sod* and *APX* genes were expressed in chloroplasts of potato plants under the control of an oxidative stress inducible promoter - *SWPA2.* These plants showed enhanced tolerance to MV and when were exposed to 42 ^{o}C for 20 h the photosynthetic activity of transgenic plants decreased by only 6%, whereas in non-transformed (NT) plants it decreased to 29% (Tang et al., 2006). The sweet potato plants expressing both *CuZnSod* and *APX* in chloroplasts using the inducible promoter have also showed a higher tolerance to MV-mediated oxidative stress and chilling stress (Lim et al., 2007). The tolerance to high and low temperature stresses was studied in tobacco plants overexpressing tomato *tAPX* gene. The overexpression of chloroplastic *APX* played a significant role in H_2O_2 detoxification and in minimizing photooxidative damage during temperature stress. The transgenic plants showed a higher photochemical efficiency of photosystem II when compared to WT plants under cold and heat stresses (Sun et al., 2010). These results suggest that the manipulation of the antioxidative mechanism in chloroplasts may be applied in the development of plants with increased tolerance to multiple environmental stresses.

High light stress

Plants exposed to excessive light can suffer photoinhibition, serious damage to the photosynthetic apparatus and degradation of photosynthetic proteins (Demmig-Adams and III, 1992). Light stress can also lead to ROS accumulation and antioxidant enzymes activation (Mittler, 2002). The APX isoenzymes responses to photooxidative stress were studied in spinach leaves during high light stress. The cAPX activity and transcripts increased during high light stress, however the protein level was not altered. The chlAPX isoforms

activities showed gradual decrease, while the other isoenzymes showed no significant variations in transcript and protein levels and activities (Yoshimura et al., 2000). In wheat, a mutant line showing decreased tAPX activity presented reduced photosynthetic activity and biomass accumulation when growing under high-light intensity, suggesting that tAPX is essential for photosynthesis (Danna et al., 2003). Single mutants of *Arabidopsis* lacking tAPX or sAPX presented higher levels of H_2O_2 and oxidized proteins than the WT plants when exposed to high light and MV stresses. The greatest effect of photooxidative stress was observed in plants lacking tAPX which had increased H_2O_2 accumulation and oxidized proteins (Maruta et al., 2010).

Double mutants deficient in two *APX* genes, one tylakoid-bound and the other cytosolic (*tylapx/apx1*), trigger different signals in *Arabidopsis* plants, such as late flowering, low protein oxidation during light stress and enhanced accumulation of anthocyanins (Miller et al., 2007). Mutants lacking a functional copy of tAPX, sAPX or both, were characterized in *Arabidopsis* under photooxidative stress during germination. The stress led to chloroplasts bleaching in *sapx* single-mutant and *tapx/sapx* double-mutant plants, while the greening process of WT and *tapx* plants was partially impaired (Kangasjarvi et al., 2008). When mature leaves of *tapx/sapx* double mutants were subjected to short-term photooxidative stress induced by high light or MV treatment, the plants showed susceptibility. The absence of chlAPXs induced alterations in the transcriptomic profile of *tapx/sapx* double-mutant plants under normal growth conditions (Kangasjarvi et al., 2008). These results indicate that the APXs isoenzymes seem to be indispensable under environmental stresses in different species, especially under light stress conditions.

In *Arabidopsis* leaves, high light treatment induced expression of cytosolic *APX2*, which expression is limited to bundle sheath cells of the vascular tissue (Fryer et al., 2003). APX1 knockout plants showed suppressed growth and development, altered stomatal responses, and induction of heat shock proteins during light stress in *Arabidopsis*. The inactivation of cytosolic APX resulted in induction of several transcripts involved in different functions. Meanwhile, the

transcripts encoding antioxidant enzymes were not elevated in transgenic APX1 plants under optimal conditions. However, during light stress, some enzymes were induced in knockout-APX1 plants (Pnueli et al., 2003).

In another study (Davletova et al., 2005) with APX1-deficient *Arabidopsis* plants it was observed that the entire chloroplastic H_2O_2-scavenging system collapsed, H_2O_2 levels increased and protein oxidation occurred in leaves subjected to a moderate light stress, suggesting that the absence of cytosolic APX1 resulted not only in accumulation of H_2O_2 but also in damage to specific proteins in leaf cells. On the other hand, rice plants double silenced for cytosolic APXs up-regulated other peroxidases, making these transgenic plants able to survive under stress, such as salt, heat, high light and MV, similar to NT plants. The antioxidative compensatory mechanism exhibited by the silenced plants was associated with increased expression of *Gpx* genes. The transcript levels of *OsCatA* and *OsCatB* and the activities of CAT and guaiacol peroxidase (GPOD; type III peroxidases) were also up-regulated. In contrast, none of the other isoforms of *OsAPX* were up-regulated under normal growth conditions. These results suggested that signaling mechanisms triggered in rice mutants could be distinct from those proposed for *Arabidopsis* (Bonifacio et al, 2011).

Drought stress

Drought stress in plants leads to severe effects such as reduction in vegetative growth and cell division. As a consequence of drought stress several changes occur inside the cell, including changes in gene expression levels, synthesis of molecular chaperones, and activation of enzymes involved in the production and removal of ROS (Mahajan and Tuteja, 2005). In two cowpea (*Vigna unguiculata*) cultivars, one drought-tolerant and the other drought-sensitive, APX activity was 60% higher in tolerant plants cultivated under control conditions. In response to drought stress, higher increase in transcript levels of cytosolic and peroxisomal *APX* genes were observed in sensitive cultivar (D'Arcy-Lameta et al., 2006). Chloroplastic *APX* genes expression was stimulated earlier in the tolerant

cultivar when subjected to drought stress. These data suggest the capacity of these enzymes to efficiently detoxify ROS at their production site (D'Arcy-Lameta et al., 2006). *APX* relative transcript levels showed distinct changes in two genotypes of wheat exposed to mild water deficit. Cytosolic *APX1* expression levels increased in both genotypes, while the cytosolic *APX2* was up-regulated only in the drought-tolerant genotype. The transcript level of thylakoid *APX* increased in drought-tolerant genotype, while the stromal *APX* showed higher expression levels in the drought-sensitive cultivar (Secenji et al., 2010).

The rice *APX* genes expression patterns were studied after 15 days drought stress. In markedly contrast with the experiments with wheat, the thylakoid *APX* transcripts (*OsAPX8*) was down-regulated in this condition, while *OsAPX1*, *OsAPX2*, *OsAPX5*, *OsAPX6* and *OsAPX7* genes were up-regulated in response to drought stress. The peroxisomal *OsAPX3* gene was not affected, while *OsAPX4* was weakly, but significantly, down-regulated by the treatment (Rosa et al., 2010). This discrepancy could be due to distinct responses of *APX* genes in different species and in different stress magnitude. In *Arabidopsis*, APX1 protein and mRNA accumulated during combination of heat and drought stress. Cytosolic *APX1*-deficient mutant accumulated more H_2O_2 and was more sensitive to stress combination than WT plants when exposed to heat and drought stress combined. In contrast, plants deficient in thylakoid or stromal/mitochondrial APXs were not more sensitive to this stress combination than *APX1*-deficient mutant or WT plants. The cytosolic *APX1* can play a key role in the acclimation of plants to combined stress such drought and heat (Koussevitzky et al., 2008). Indeed, when the overexpression of cytosolic *APX* in tobacco was studied, it showed that the overexpression protected the plant from several oxidative stresses, including drought and polyethylene glycol-induced stress (Badawi et al., 2004).

Plants overexpressing other antioxidant enzymes in different species showed increased tolerance to various stresses, including drought resistance. The overexpression of a *Populus* peroxisomal ascorbate peroxidase (*PpAPX*) gene in transgenic tobacco improved drought resistance in these plants (Li et al., 2009). The overexpression of tomato (*Solanum lycopersicum*) thylakoid-bound *APX*

(*StAPX)* gene in tobacco plants enhanced the tolerance of these plants to salt and osmotic stress (Sun et al., 2010).

Heavy metals

The soil contamination with heavy metals is a serious environmental problem that limits crop production. Exposure at higher concentrations of heavy metals can increase the production of ROS and change antioxidant response (Gratão et al., 2005). The treatment with cadmium in pea (*Pisum sativum* L.) plants changed enzymatic and non-enzymatic antioxidant defenses, however, APX activity or accumulation of its transcripts was not significantly different (Romero-Puertas et al., 2007). However, it was observed that in coffee cells, the activity of APX increased in the lower cadmium concentration. On the other hand, APX activity was not detectable in cells subjected to the higher cadmium concentration after 24 h of treatment (Gomes-Junior et al., 2006).

The increase of APX activity was also observed in response to other heavy metals such as aluminum (Sharma and Dubey, 2007). In rice, the transcript levels of all *OsAPX* genes, except *OsAPX6*, were significantly increased after eight hours of 20 ppm aluminum exposure (Rosa et al., 2010). In pea plants, the *cAPX* expression increased in the shoots under aluminum treatment, however, APX activity presented significant decline at 10 µM aluminum in roots and shoots after 24 and 48 h of stress, but at 50 µM aluminum treatment, APX activity did not show significant changes (Panda and Matsumoto, 2010).

Transgenic rice plants double silenced for *APX1* and *APX2* (APX1/2s plants) exhibited normal development and enhanced tolerance to a toxic concentration of aluminum (Rosa et al., 2010). In bean plants, the cytosolic APX expression at mRNA and protein levels was induced in leaves of de-rooted plants in response to iron overload. Likewise, transgenic tobacco plants with suppressed cytosolic APX levels were more sensitive to iron application than WT plants (Pekker et al., 2002). In coffee cells, the nickel treatment increased APX activity

rapidly, although the activity trends were slightly different between the two nickel concentrations tested (0.05 mM and 0.5 mM) (Gomes-Junior et al., 2006).

Transgenic tall fescue plants expressing the *CuZnSOD* and *APX* genes in chloroplasts were submitted to copper, cadmium or arsenic treatment. Of the metals tested, copper and cadmium increased SOD and APX activities in control and transgenic plants, however, the increase was greater in transgenic plants. Whereas, in leaves exposed to arsenic treatment, both enzymes exhibited less activity when compared to other treatments and no significant differences were observed between control and transgenic plants (Lee et al., 2007). These results emphasize the important role of APX and other antioxidant enzymes in H_2O_2 scavenging under toxic metals levels in the soil.

CONCLUSIONS

Ascorbate peroxidase is a key enzyme regulating ROS levels acting in different subcellular compartments (Fig.2). The *APX* encoding genes expression is differentially modulated by several abiotic stresses in different plant species. All the data collected so far firmly indicate that APX play an important and direct role as a protective element against adverse environmental conditions. On the other hand, the diverse effect of knockdown or knockout of different *APX* genes in the plant growth, physiology and antioxidant metabolism have showed that APX may also regulate redox signaling pathways involved in plant development. These results point to the importance and the complexity of APX relationship with other antioxidants in fine tuning the vegetal antioxidant metabolism.

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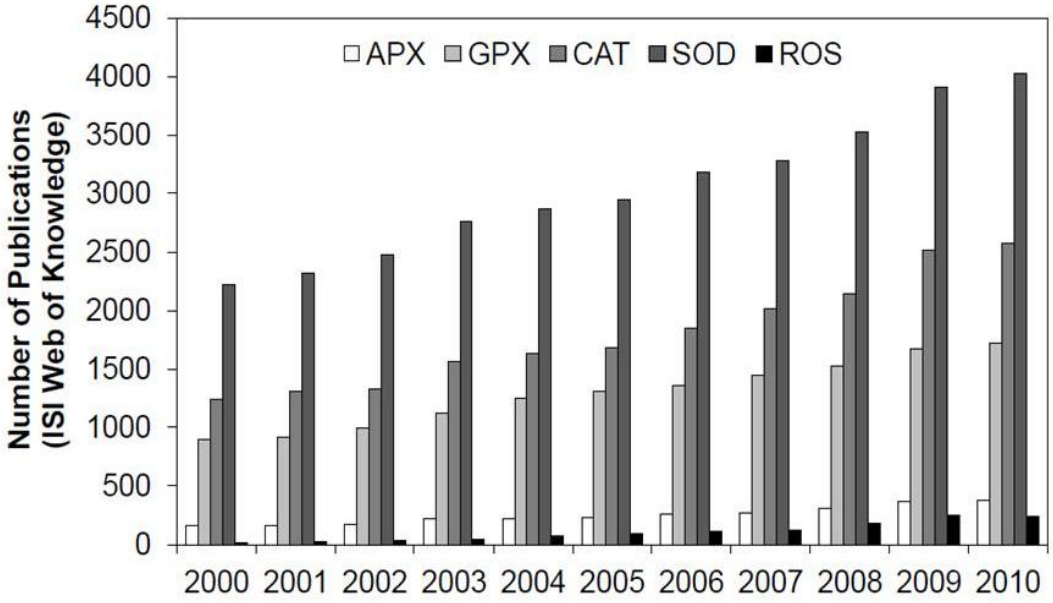

Figure 1. Number of publication evolution addressing antioxidant enzymes in plants in the last ten years. Ascorbate peroxidase (APX), gluthatione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD) enzymes, reactive oxygen species (ROS).

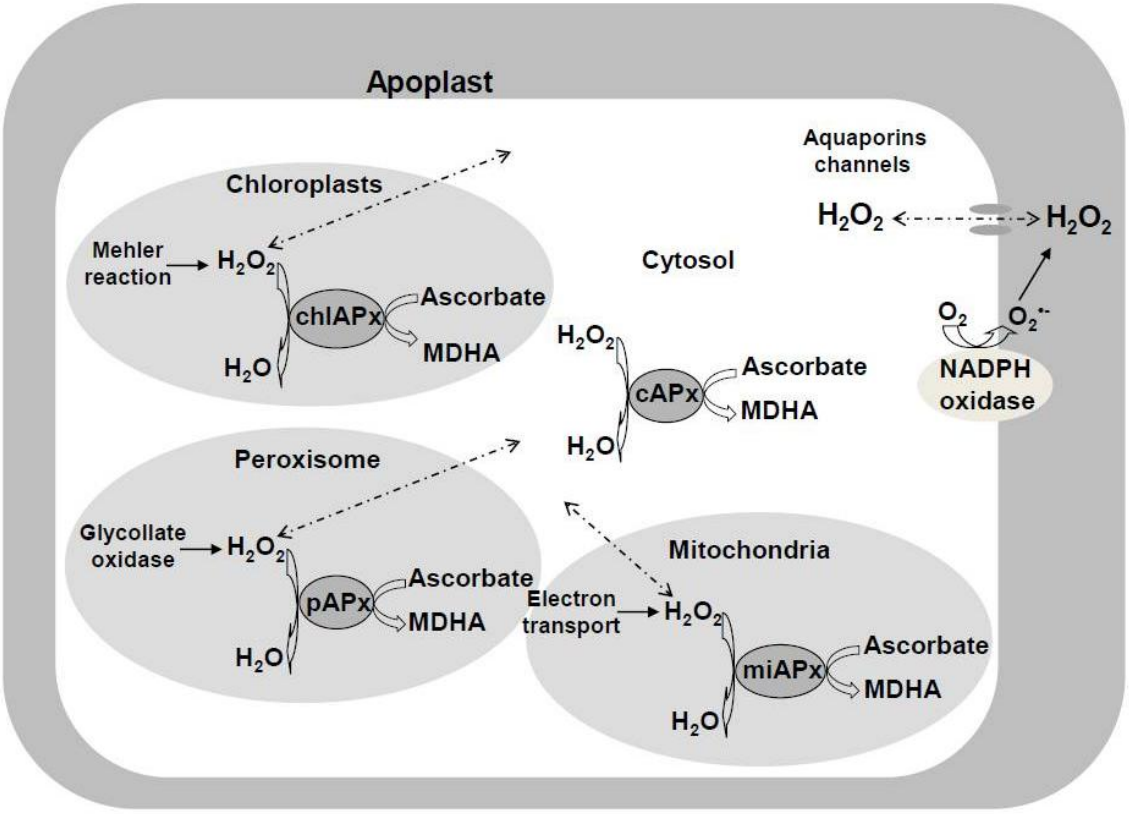

Figure 2. APX enzymes and the elimination of ROS excess in different subcellular compartments. H_2O_2 is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes. Abiotic and biotic stresses enhance H_2O_2 and chloroplastic ascorbate peroxidase (chlAPX), microbody ascorbate peroxidase (mAPX), cytosol ascorbate peroxidase (cAPX) and mitochondria ascorbate peroxidase (miAPX) enzymes can act eliminating ROS excess in different subcellular compartments. The plasmatic membrane-NADPH oxidases also generate H_2O_2, which can cross membranes through aquaporins channels. Ascorbate peroxidase (APX), oxygen (O_2), superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), water (H_2O), monodehydroascorbate (MDHA).

CAPÍTULO 2

Localização subcelular dos produtos dos genes de *OsAPX* em arroz

INTRODUÇÃO

A atividade de APX foi descrita pela primeira vez em 1980 (Shigeoka et al., 1980), desde então uma série de estudos vêm demonstrando a importância da enzima APX em diversos organismos. As APXs são encontradas em uma variedade de espécies de plantas codificadas por pequenas famílias gênicas. APX existe em várias isoformas e estão distribuídas em diferentes compartimentos na célula (Panchuk et al., 2002; Shigeoka et al., 2002; Chew et al., 2003). A presença de peptídeos de direcionamento organela-específica e de domínios transmembrana encontrados nas regiões N- e C-terminal das proteínas determina a localização subcelular das diferentes isoformas de APX (Shigeoka et al., 2002; Teixeira et al., 2004; Teixeira et al., 2006). Além disso, as propriedades bioquímicas da APX como massa molecular, especificidade pelo substrato, pH ótimo e estabilidade na ausência de ascorbato estão correlacionadas com a localização subcelular dessas proteínas (Ishikawa et al., 1998). Isoformas solúveis são encontradas no citosol, mitocôndria e no estroma do cloroplasto. Enquanto que isoformas ligadas às membranas encontram-se nos peroxissomos e no tilacóide do cloroplasto (Shigeoka et al., 2002; Teixeira et al., 2004; Teixeira et al., 2006).

Análises filogenéticas das famílias de APX em diferentes organismos revelaram que as isoformas de um mesmo compartimento subcelular possuem uma origem comum. Essas análises demonstraram que isoformas de um mesmo compartimento celular são mais semelhantes entre si do que quando comparadas a isoformas de diferentes compartimentos celulares de um mesmo organismo (Teixeira et al., 2004; 2005). Entre as monocotiledôneas, o maior número de izoenzimas APX é encontrado em trigo (*Triticum aestivum*), treze sequências encontram-se depositadas no banco de dados Peroxibase (http://peroxibase.isb-

sib.ch), essas são divididas em citosólicas, tilacoidal, estromal e peroxissomal APXs.

Em arroz a presença de oito isoformas de OsAPX foi previamente descrita. Todas essas isoformas possuem um sítio ativo e um sítio de ligação ao heme, característico das heme-peroxidases (Figura 1) (Teixeira et al., 2004). Inicialmente foram identificadas por análises *in silico*, duas isoformas citosólicas, duas peroxissomais e quatro isoformas cloroplastídicas. As isoformas de OsAPX identificadas no genoma do arroz apresentam a combinação de domínios apropriados para a predita localização subcelular dessas proteínas (Figura 1) (Teixeira et al., 2004). A estrutura gênica e as principais características de todos os membros da família de OsAPX em arroz são apresentadas na Tabela 1.

Posteriormente, a localização subcelular de duas isoformas, OsAPX3 e OsAPX6 foram determinadas *in vivo*, porém em sistema heterólogo utilizando células BY-2 de tabaco (Teixeira et al., 2006). No presente trabalho a localização subcelular das isoformas de OsAPX em arroz foi determinada experimentalmente *in vivo* em sistema homólogo.

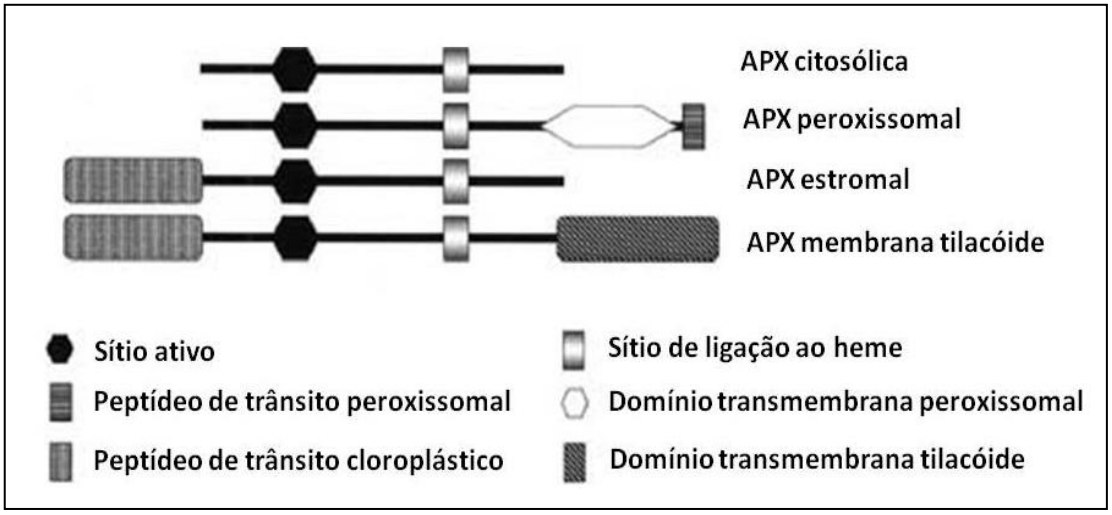

Figura 1. Estrutura das proteínas das isoformas de OsAPX em arroz. Comparação da estrutura primária das proteínas de OsAPX dos distintos compartimentos subcelulares. Os diferentes domínios são representados por diferentes símbolos (Adaptado de Teixeira et al., 2004).

Tabela 1. Estrutura gênica e principais características da família de OsAPX (Adaptado de Teixeira et al., 2004).

	Cromossomo	Número de exons	Número de introns	cDNA/ORF (pb)	Proteina (aa)	PM (kDa)	Número de acesso NCBI cDNA	Número de acesso NCBI proteina	Localização Subcelular Predita
OsAPX1	3	9	8	983/753	250	27.2	D45423	BAA08264	Citosol
OsAPX2	7	9	8	1160/756	251	27.1	AB053297	BAA20889	Citosol
OsAPX3	4	9	8	1060/876	291	32.0	AY382617	AAQ88105	Peroxissomo
OsAPX4	8	9	8	1244/876	291	31.7	AK070842	-	Peroxissomo
OsAPX5	12	11	10	1129/963	320	34.8	AK073910	-	Cloroplasto
OsAPX6	12	11	10	1288/930	309	33.5	AK061107	-	Cloroplasto
OsAPX7	4	12	11	1392/1080	359	38.3	AB114855 AK103344	BAC79362	Cloroplasto
OsAPX8	2	12	11	1698/1497	478	51.2	AK070842	BAC79363	Cloroplasto

Nota: cDNA – DNA complementar, ORF – fase aberta de leitura, do inglês “Open reading frame”, pb – pares de bases, aa – aminoácidos, PM – peso molecular, kDA – kilodalton e NCBI – National Center for Biotechonology Information.

MATERIAL E MÉTODOS

Construção dos vetores para localização subcelular dos produtos dos genes de *OsAPX* em arroz

As sequências completas de cDNA das OsAPXs foram obtidas no banco de dados NCIB (National Center for Biotechnology Information) (código de acesso no NCBI: *OsAPX1* - D45423, *OsAPX2* - AB053297, *OsAPX3* - AY382617, *OsAPX4* - AK070842, *OsAPX5* - AK073910, *OsAPX6* - AK061107, *OsAPX7* - AK103344 e *OsAPX8* - BAC79363). Posteriormente foram projetados oligonucleotídeos iniciadores específicos para cada sequência de cDNA de *OsAPX* (Tabela 2). Estes foram utilizados para amplificar o cDNA completo de *OsAPX* por PCR. O produto do PCR foi clonado no plasmídeo pDONOR^{TM}201 (Invitrogen). Esses foram denominados pDONOR-OsAPX1, pDONOR-OsAPX2, pDONOR-OsAPX3, pDONOR-OsAPX4, pDONOR-OsAPX5, pDONOR-OsAPX6 e

pDONOR-OsAPX7. Em relação ao cDNA completo de *OsAPX8* este não foi possível amplificar.

A localização subcelular dos produtos gênicos de OsAPX foi realizada por expressão transiente em protoplastos de arroz. Os cDNAs de OsAPX foram clonados em fusão traducional com a proteína YFP (Yellow Fluorescent Protein) com auxílio do sistema de clonagem Gateway. As proteínas OsAPX5, OsAPX6 e OsAPX7 foram clonadas em fusão com a sequência codificante da proteína YFP na sua porção N-terminal (35S-OsAPX5::YFP, 35S-OsAPX6::YFP e 35S-OsAPX7::YFP), pois estas possuem um peptídeo de trânsito para o cloroplasto ou mitocôndria na N-terminal das proteínas, desse modo, os oligonucleotídeos iniciadores específicos que foram projetados para as fusões na N-terminal da proteína YFP foram projetados sem o códon de término da tradução. O plasmídeo utilizado foi pART7-HA-YFP (Galvan-Ampudia & Offringa, 2007), o qual expressa a proteína de fusão sob o controle do promotor 35S do CaMV e permite a clonagem na porção N-terminal da proteína YFP.

As proteínas OsAPX3 e OsAPX4 foram clonadas na C-terminal da proteína YFP (35S-YFP::OsAPX3, 35S-YFP::OsAPX4) e recombinadas no vetor p2YGW7 (Karimi et al., 2005). Essas proteínas possuem um peptídeo de trânsito na porção C-terminal o qual leva ao direcionamento da proteína para o peroxissomo (Teixeira et al., 2004). Já as proteínas OsAPX1 e OsAPX2 foram clonadas tanto na porção N-terminal quanto na C-terminal da proteína YFP (35S-OsAPX1::YFP, 35S-OsAPX2::YFP, 35S-YFP::OsAPX1, 35S-YFP::OsAPX2). Essas duas proteínas não possuem peptídeo de direcionamento para uma organela (Teixeira et al., 2004), sendo assim, foi possível realizarmos clonagens nas duas porções da proteína YFP para OsAPX1 e OsAPX2, desse modo possibilitando observar se a localização subcelular da proteína é alterada dependendo do sentido da fusão da proteína na porção N ou C-terminal da YFP. O cassete 35S-GFP (Galvan-Ampudia & Offringa, 2007) foi utilizado como controle positivo da localização.

As reações de recombinação dos plasmídeos pDONOR-OsAPX foram realizadas seguindo as instruções do fabricante onde 2 µL de cada plasmídeo pDONOR-OsAPX foi recombinado com 2 µL do vetor pART7-HA-YFP e/ou

p2YGW7 e adicionado 2 µL de tampão TE e misturado levemente por pipetagem. Em seguida 1 µL da enzima clonase foi adicionado a cada reação. As reações permaneceram 2 h a 37 ^{o}C. Após esse período, 1 µL de proteinase K foi adicionado em cada reação e essas foram incubadas a 37 ^{o}C por 10 minutos. Posteriormente 1 a 2 µL da reação foram usados para transformação de *E. coli* por eletroporação.

Transformação de *Escherichia coli*

Os vetores de localização subcelular de *OsAPX* foram clonados em células competentes de *E. coli DH5α*. As células competentes de *E. coli* para transformação por eletroporação foram produzidas conforme o protocolo descrito em Sambrook et al (1989). Para a transformação por eletroporação em *E. coli*, 1 a 2 µL do produto da recombinação foram adicionados a 80 µL de células competentes. A mistura foi eletroporada em cubeta de 0,2 cm sob pulso elétrico de 2,500 V. (Eletroporador Bio Rad, Gene Pulser® II). A seguir, 300 µL de meio LB líquido (Invitrogen) foram adicionados às células.

O material foi então transferido para um tubo de microcentrífuga de 1,5 mL e incubado por 1 h a 37 ºC sob agitação constante. Volumes de 50 e 250 µL da cultura contendo as bactérias foram plaqueadas em duas placas com meio LB sólido (Invitrogen) contendo o antibiótico ampicilina (150 µg/mL) para seleção de bactérias transformadas. As placas foram mantidas em estufa a 37 ºC por 16 horas. As colônias de bactérias que cresceram no meio contendo o antibiótico ampicilina foram selecionadas e o DNA plasmidial foi extraído. As transformações das colônias de bactérias selecionadas foram confirmadas por PCR utilizando os oligonucleotídeos iniciadores específicos para a construção.

Extração de DNA plasmidial

Após a clonagem dos vetores de localização subcelular de OsAPX em *E. coli* as colônias isoladas de bactérias foram selecionadas e lançadas em 4 mL de

meio de cultura LB liquído contendo o antibiótico ampicilina (150 µg/mL). Após o período de crescimento da cultura de 16 h a extração de plasmídeos de *E. coli* foi realizada pelo processo de lise alcalina conforme descrito por Sambrook et al. (1989). Células de 1,5 mL da suspensão de bactérias foram concentradas por centrifugação em 13,000 x *g* por 1 minuto. Após o descarte do sobrenadante, 200 µL da solução I (Tris-HCL 25 mM pH 8,0; EDTA 10 mM; glicose 25 mM) foram adicionadas às células e a mistura foi homogeneizada em vortex.

Em seguida, a solução II (200 µL) contendo NaOH 0,2 N e SDS 1% foi misturada lentamente por inversão à mistura anterior. Posteriormente 200 µL da solução III (NaAC 4,5 M pH 5,2) foram adicionados e homogeneizados as outras por inversão. Após centrifugação por 5 minutos a 13,000 x *g*, 600 µL do sobrenadante foi transferido para novo tubo. Os plasmídeos foram então precipitados utilizando 600 µL de isopropanol e centrifugação por 20 minutos em 13,000 x *g*. O sobrenadante foi descartado e o precipitado lavado com 350 µL de etanol 70%. A mistura foi centrifugada por 5 minutos a 13,000 x *g*. O sobrenadante foi descartado e o precipitado foi seco em estufa a 37 ^{o}C. O DNA plasmidial foi resuspendido em 50 µL de água ultra pura autoclavada.

Reação em cadeia da DNA polimerase (PCR)

As reações de PCR foram realizadas para amplificar o cDNA das OsAPXs e para a confirmação da clonagem em *E. coli* dos mesmos. Nessas reações foram utilizados os oligonucleotídeos iniciadores específicos para as construções de estudo da localização subcelular das proteínas de OsAPX (oligonucleotídeos denominados OsAPX1, OsAPX2, OsAPX3, OsAPX4, OsAPX5, OsAPX6 e OsAPX7) (Tabela 2).

Para a reação de amplificação, foram adicionados 2 µL de cDNA de arroz (cv. Nipponbare subespécie *Japonica*) e/ou DNA plasmidial, 0,5 µL de dNTP a 10 mM, 2 µL de tampão de PCR 10X concentrado (Tris/HCL a 100 mM, (pH 8,0), KCl a 500 mM), 1 µL de $MgCl_2$ a 10 mM, 1 µL de cada oligonucleotídeo iniciador a 10 mM, 0,2 µL de Taq DNA-polimerase (5 U/µL) e 12,3 µL de água ultrapura estéril.

As condições utilizadas na amplificação dos cDNAs e/ou DNA plasmidial foram: 94 ^{o}C por 5 minutos, seguido de 35 ciclos de desnaturação (94 ^{o}C por 1 min), anelamento 55 ^{o}C, (1 min) e extensão (72 ^{o}C por 1 min). O último ciclo foi seguido de uma extensão final de 5 minutos a 72 ^{o}C. A amplificação e o tamanho dos fragmentos de DNA foram confirmados por eletroforese em gel de agarose 1 a 2% corados com GelRed^{TM} (Biotium, Inc.).

Tabela 2. Oligonucleotídeos iniciadores específicos utilizados para amplificar as sequências de cDNA de OsAPX e confirmação da clonagem em *E. coli* por PCR.

OsAPX1	F 5'-ATGGCTAAGAACTACC- 3' R 5'-AGCATCAGCGAACCCCAG- 3'
OsAPX2	F 5'-ATGGGCAGCAAGTC-3' R 5'-TTCCTCAGCAAATCCCAG-3'
OsAPX3	F 5'-ATGTCGGCGGCGCCGGTAG-3' R 5'-TTAGCCAAGCCTCTTGTT-3'
OsAPX4	F 5'-ATGGCCGCCCCGGTCGTG-3' R 5'-TTACTTGCTCTTCTTAGAA -3'
OsAPX5	F 5'-ATGGCCGTCGTGCA-3' R 5'-TTCAAGTGAAATACCCTTTG-3'
OsAPX6	F 5'-ATGGCCGTCGTCCA-3' R 5'-TTCCAGTGAAATACCCTTT-3'
OsAPX7	F 5'-ATGGCGGCCCAGCG-3' R 5'-ACCGTCCAACGTGAATCC-3'

Isolamento de protoplastos de arroz e transfecção das células

Sementes de arroz da cultivar Nipponbare, subespécie japônica foram descascadas e, em seguida, desinfestadas. A desinfestação consistiu de uma lavagem com etanol 70% por 1 minuto. Após as sementes foram lavadas com água destilada por três vezes, em seguida as sementes foram mergulhadas em solução de alvejante a 50% (hipoclorito de sódio 2%) por 30 minutos sob agitação. Sob condições assépticas, essas sementes foram lavadas diversas vezes em água estéril e plaqueadas em meio MS (Sigma-Aldrich) e crescidas a 28 ^{o}C.

O isolamento dos protoplastos foi realizado seguindo o protocolo descrito por Chen et. al (2006). Plantas com aproximadamente 15 a 20 dias de idade foram usadas para isolar os protoplastos. As mesmas foram cortadas em pequenos fragmentos (caule e folha) de aproximadamente 0,5 mm. Foram usadas aproximadamente 60 plantas para o isolamento de um número suficiente de células. O material vegetal foi colocado em um frasco contendo 10 ml de solução de enzima ("Macerozyme R10 e Cellulase R10", SERVA) (Chen et al., 2006) o qual foi vedado com filme plástico e aplicado vácuo por 1 hora para infiltração da solução de enzima nos tecidos. Após esse período, a solução foi incubada por 4 horas no escuro sob agitação leve (~40 rpm) à temperatura ambiente. Em seguida, a solução de enzima foi removida, e foi adicionado ao material vegetal 25 mL de meio W5 (Chen et al., 2006) para liberar os protoplastos. O material foi incubado por mais uma hora sob agitação (~80 rpm). Posteriormente, o meio contendo os protoplastos foi filtrado através de um filtro de nylon de 35 µm (BD Falcon).

A solução foi distribuída em quatro tubos de 15 mL e centrifugada a 1000 rpm por 5 minutos para coletar os protoplastos. Em seguida, o meio W5 foi removido e foram adicionados a cada tubo contendo os protoplastos 0,5 mL de meio de suspensão de protoplastos (Chen et al., 2006). Em seguida foi feita a contagem do número aproximado de células utilizando 10 µL de suspensão de protoplastos em câmera de Neubaer e visualizados no microscópio. A concentração de células recomendadas é de 1,5 a 2.5 x 10^6 células/mL (Chen et al., 2006). Após a resuspensão dos protoplastos no volume adequado de meio de suspensão foram preparados tubos de 2 mL contendo 10 µg de DNA plasmidial (1 µg/1 µL) de cada construção para transformação conforme Tao et al. (2002). Em seguida, foram adicionados 200 µL (1.5 ~2.5 x 10^6 células/mL) de células de protoplastos em meio de suspensão. Após foi adicionado 250 µL de solução de PEG 40%. A mistura foi incubada por 10 minutos a temperatura ambiente. Em seguida, foi adicionado 1 mL de meio W5 no tubo para diluir o PEG e os protoplastos transfectados com o DNA foram mantidos na estufa incubadora no escuro a 28 ^oC por 24 horas.

A análise das células transfectadas foi realizada no microscópio confocal de fluorescência (Olympus FluoView 1000) e a captura das imagens foi realizada no aumento de 60X usando o software Olympus Fluoview FV10-ASW. O marcador DAPI (4´,6-diamino-2-fenilindole) (Sigma) foi utilizado para marcar o núcleo das células. Este foi adicionado aos protoplastos numa concentração final de 0,2 mg/mL e incubado no escuro por 10 minutos antes da visualização no microscópio. A excitação do DAPI foi de 358 nm e a emissão máxima de 461 nm. Para marcar as mitocôndrias foi utilizado o marcador Mito Tracker Red FM (Invitrogen) numa concentração final de 300 nM e as células de protoplastos foram incubadas por 20 minutos antes da visualização no microscópio. A excitação do Mito Tracker Red FM foi realizada a 543 nm e a detecção da emissão a 590-640 nm. A excitação da auto-fluorescência da clorofila foi feita a 635 nm com a detecção da emissão a 670-700 nm. A excitação da YFP realizada a 488 nm e a emissão detectada entre 510-528 nm. A excitação da GFP foi de 488 nm e a emissão a 510-550 nm.

RESULTADOS E DISCUSSÃO

Neste capítulo, são apresentados os resultados das localizações subcelulares referentes às proteínas OsAPX1, OsAPX2, OsAPX3 e OsAPX4. As localizações das proteínas OsAPX5, OsAPX6 e OsAPX7 estão descritas no capítulo 3.

OsAPX1 e OsAPX2

Análises *in silico* em programas de predição de localização subcelular revelaram que as proteínas OsAPX1 e OsAPX2 não possuem nenhum sinal de trânsito nem domínios transmembrana específicos para uma organela, indicando que os genes *OsAPX1* e *OsAPX2* codificam isoformas citosólicas (Figura 1) (Teixeira et al., 2004). A localização subcelular das proteínas OsAPX1 e OsAPX2 em protoplastos de arroz com fusões tanto no N-terminal (35S-OsAPX1::YFP,

35S-OsAPX2::YFP) quanto no C-terminal (35S-YFP::OsAPX1, 35S-YFP::OsAPX2) da proteína YFP demonstraram que a fluorescência de YFP foi igualmente acumulada no citosol (Figura 2B e D). Além disso, a fluorescência de YFP foi também observada no núcleo das células transformadas. Protoplastos corados com DAPI e visualizados no microscópio confocal confirmaram a localização da fluorescência de YFP no núcleo das células (Figura 2E).

Somente foi possível visualizar células intactas e marcadas com o marcador nuclear DAPI para a fusão 35S-OsAPX2::YFP. No entanto, para fusão 35S-OsAPX1::YFP foram identificadas várias células onde o marcador DAPI co-localizou no núcleo com a fluorescência do YFP (dados não mostrados), porém essas células não estavam intactas. Teixeira (2005) relatou em sua dissertação de mestrado que análises da fusão da proteína OsAPX1 com a proteína GFP (Green Fluorescent Protein) demonstraram que a fluorescência de GFP também foi observada no citosol e no núcleo de células BY-2 de tabaco transformadas.

Nossos resultados estão de acordo com aqueles obtidos pelas análises *in silico* da família de APX em arroz, onde OsAPX1 e OsAPX2 foram preditas estarem localizadas no citosol (Teixeira et al., 2004) e também com os resultados obtidos por Teixeira (2005) que relata a localização subcelular da proteína citosólica APX1 também no núcleo de células de tabaco. No entanto, as análises *in silico* de predições das proteínas de OsAPX em arroz (Teixeira et al., 2004) não demonstraram que OsAPX1 e OsAPX2 possam também estar localizadas no núcleo celular, pois nenhum sinal de localização nuclear foi encontrado nessas proteínas. Sendo assim, experimentos adicionais são necessários para comprovar a localização subcelular das proteínas OsAPX1 e OsAPX2 também no núcleo celular em arroz. Anticorpos específicos para a APX1 e APX2 já estão disponíveis e poderão ser utilizados para ensaios de imunolocalização.

Em *Arabidospsis*, os genes *APX1* e *APX2* também codificam isoformas localizadas no citosol (Panchuk et al., 2002), no entanto, não existem relatos na literatura da atividade de APX no núcleo celular de outras espécies vegetais até o momento. Entretanto, há vários estudos demonstrando que as APXs citosólicas exercem importantes funções no metabolismo antioxidante em plantas removendo ERO no citosol para manter a homeostase celular. Em *Arabidopsis* mutantes

deficientes em *APX1* apresentaram menor taxa fotossintética, crescimento mais lento e atraso no florescimento em condições normais (Pnueli et al., 2003). Em arroz, plantas silenciadas simultaneamente para ambos os genes *OsAPX1* e *OsAPX2* mostraram maior tolerância a concentrações tóxicas de alumínio. Por outro lado, plantas silenciadas para o gene *OsAPX1* ou *OsAPX2* apresentaram fortes alterações fenotípicas (Rosa et al., 2010). Além disso, duplo mutantes em *OsAPX1* e *OsAPX2* tiveram expressão aumentada dos genes de outras peroxidases (Bonifacio et al., 2011) e alteração na expressão de genes de vias metabólicas específicas, notadamente as relacionadas com fotossíntese e fotorrespiração, demonstrando a importância das APXs citosólicas na homeostase redox e seu efeito na regulação da expressão gênica (Ribeiro et al., 2012).

Poucos relatos encontram-se na literatura sobre a atividade das enzimas antioxidantes no núcleo celular vegetal. Em cevada, a proteína 1-Cys PER1, a qual é uma peroxirredoxina e têm atividade antioxidante, foi localizada no núcleo de embriões em desenvolvimento e no núcleo das células de aleurona, além de um fraco sinal encontrado no citoplasma (Stacy et al., 1999). Da mesma forma, em couve (*Brassica campestris L. ssp. Pekinensis*) e *Arabidopsis* uma proteína peroxirredoxina também foi localizada no citosol e núcleo celular (Haslekas et al., 2003; Kim et al., 2012). Os autores sugerem uma função de proteção dos ácidos nucléicos contra ERO dessas proteínas com função antioxidante que se localizam no núcleo.

Em animais, uma proteína antioxidante SOD nuclear parece proteger o DNA genômico de ERO atuando como um fator de eliminação de $O_2^{\bullet-}$ no núcleo. Sabe-se que o DNA é clivado através de ataque por ERO, dessa forma o DNA genômico pode ser danificado como resultado da acumulação de $O_2^{\bullet-}$. É provável que ERO, incluindo o $O_2^{\bullet-}$, possa interagir com íons de cobre ligados ao DNA e formar $OH^{\bullet}$ via reação de “Fenton”, uma vez que os íons de cobre são importantes componentes da cromatina e potencialmente podem ser um fator no dano de base do DNA. Assim, SOD pode estar envolvida na proteção do DNA genômico (Kaneto et al., 1994; Ookawara et al., 2002). Entretanto, os estudos sobre a função exercida por proteínas antioxidantes que se encontram no núcleo celular necessitam ser mais explorados.

Nossos resultados indicam um papel de proteção desempenhado pelas enzimas OsAPX1 e OsAPX2 no núcleo. No entanto, não se pode descartar a possibilidade de que essas proteínas possam estar atuando como reguladores da expressão gênica, ou diretamente através da modificação redox de proteínas reguladoras, ou indiretamente através da modulação dos níveis de peróxido de hidrogênico, o qual, sabidamente, pode atuar como molécula sinalizadora para a regulação da expressão gênica.

OsAPX3 e OsAPX4

As isoformas peroxissomais de APX apresentam um domínio transmembrana hidrofóbico rico em resíduos de alanina e valina, seguido por um domínio carregado positivamente contendo cinco resíduos presentes no C-terminal da proteína (Mullen & Trelease, 2000). Os produtos proteicos de *OsAPX3* e *OsAPX4* de arroz possuem essas mesmas características e foram preditos como isoformas peroxissomais (Figura 1) (Teixeira et al., 2004). As proteínas de fusão 35S-YFP::OsAPX3 e 35S-YFP::OsAPX4 foram construídas de modo que as proteínas OsAPX3 e OsAPX4 se localizassem na extremidade C-terminal da fusão para evitar que os domínios de direcionamento para o peroxissomo estivessem inacessíveis na conformação final da proteína recombinante.

As imagens de fluorescência da proteína de fusão 35S-YFP::OsAPX3 e 35S-YFP::OsAPX4 em protoplastos de arroz mostram que OsAPX3 e OsAPX4 estão localizadas nos peroxissomos e no retículo endoplasmático (Figura 2G e J). Esses resultados estão de acordo com as predições iniciais *in silico*, onde os produtos proteicos dos genes *OsAPX3* e *OsAPX4* foram localizados nos peroxissomos em arroz (Teixeira et al., 2004) e com os dados da localização subcelular *in vivo* obtidos por Teixeira et al. (2006). Estudos prévios demonstram que as isoformas de APX peroxissomais são ordenadas indiretamente para o peroxissomo via uma rede de membranas reticulares que são compostas por um sub-domínio do retículo endoplasmático rugoso, denominado pER (“peroxisomal endoplasmic reticulum”) (Mullen & Trealease, 2000, 2006; Teixeira et al., 2006).

As imagens de fluorescência das fusões 35S-YFP::OsAPX3 e 35S-YFP::OsAPX4 seguem um padrão similar ao que foi encontrado por Teixeira et al. (2006). A fluorescência foi detectada em uma estrutura reticular/circular ao redor dos cloroplastos e difusamente no citosol de protoplastos de arroz (Figura 2), similar ao que foi observado em células BY-2 de tabaco (Teixeira et al., 2006). Sendo assim, nossos resultados confirmam, em arroz, que OsAPX3 e OsAPX4 são isoformas peroxissomais, sendo as mesmas transportadas para os peroxissomas via sub-domínio do reticulo endoplasmático rugoso, conforme também já observado em *Arabidopsis* e em algodão (Mullen et al., 1999; Mullen & Trealease, 2000, 2006; Lisenbee et al., 2003; Narendra et ., 2006).

As APXs peroxissomais exercem importantes funções protegendo as células vegetais, atuando na eliminação dos níveis tóxicos de ERO. Além disso, estão envolvidas em cascatas de sinalização que iniciam em resposta a estímulos externos (Shigeoka et al., 2002; Mullen & Trealease, 2006). Uma APX peroxissomal de cevada foi super-expressa em *Arabidopsis* e as plantas transgênicas foram mais tolerantes a estresse de calor. Além disso, o gene de *APX* peroxissomal de cevada aumentou seus transcritos em resposta aos tratamentos de calor, sal e ácido abscísico (Shi et al., 2001). Por outro lado, mutantes deficientes em uma APX peroxissomal de *Arabidopsis* não apresentaram diferenças morfológicas e de biomassa nem mesmo quando as plantas foram submetidas a estresses abióticos. Nesse caso, possivelmente outra isoforma peroxissomal de APX em *Arabidopsis* pode ter compensado a deficiência de APX, uma vez que tenham funções redundantes no metabolismo antioxidante de *Arabidopsis* (Narendra et al., 2006).

Em arroz, a deficiência dessas isoformas de OsAPX geraram plantas com floração atrasada e mais sensíveis à senescência (Ribeiro, 2012). Entretanto, os estudos com as isoformas peroxissomais de APX em plantas ainda são pouco explorados, quando comparados com os das isoformas citosólicas e cloroplastídicas.

Este trabalho permitiu confirmar em protoplastos de arroz a localização subcelular de duas APX citosólicas e duas APX peroxissomais, o que contribuirá

para o entendimento da função desses genes nas respostas da planta a diferentes estresses. As proteínas citosólicas de arroz, APX1 e APX2, parecem possuir duplo direcionamento para o citosol e núcleo, entretanto mais experimentos utilizando outras técnicas são necessários para confirmar esses dados. Uma vez confirmado, este resultado terá forte impacto para estudos posteriores visando a determinação da função dessas proteínas no metabolismo vegetal.

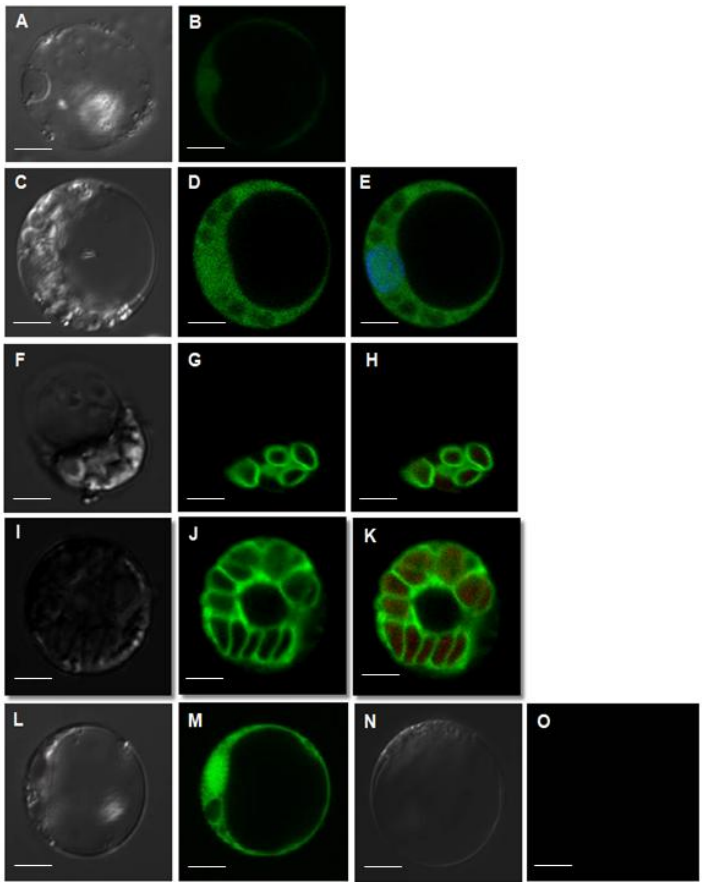

Figura 2. Localização subcelular das proteínas de fusão OsAPX1::YFP, OsAPX2::YFP, YFP::OsAPX3 e YFP::OsAPX4 em protoplastos de arroz. (A, C, F, I, L e N) Imagem de contraste de interferência de células de protoplastos de arroz transformadas. (B, D, G, J e M) Fluorescência da YFP de células de protoplastos de arroz expressando a proteína fusão (B) OsAPX1::YFP citosol/núcleo, (D) OsAPX2::YFP citosol/núcleo, (G) YFP::OsAPX3 peroxissomo, (J) YFP::OsAPX4 peroxissomo e (M) cassete GFP controle positivo da localização, proteína GFP citosol/núcleo. (E, H, e K) Sobreposição de imagens (E) fluorescência de OsAPX2::YFP com a fluorescência do marcador de núcleo celular DAPI, (H) fluorescência de YFP::OsAPX3 com a fluorescência da clorofila, (K) fluorescência de YFP::OsAPX4 com a fluorescência da clorofila. (O) Controle negativo. Todas as barras de escala representam 10 µm.

CAPÍTULO 3

A double knockdown of chlAPX in rice altered photochemical and biochemical phases of photosynthesis, revealing the regulatory role of chlAPX in the photosynthetic process

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A double knockdown of chlAPX in rice altered photochemical and biochemical phases of photosynthesis, revealing the regulatory role of chlAPX in the photosynthetic process

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ABSTRACT

The inactivation of the chloroplast ascorbate peroxidases (APXs) has been thought to limit the efficiency of the water-water cycle and photo-oxidative protection under stress conditions. In this study, we have generated double knockdown rice (*Oryza sativa* L.) plants in both *OsAPX7* and *OsAPX8* genes,

which encode chloroplastic APXs (chlAPXs). By employing an integrated approach involving gene expression, proteomics, biochemical and physiological analyses of photosynthesis, we have assessed the role of chlAPXs in the regulation of photosystem I (PSI) and photosystem II (PSII) and photosynthesis of rice plants exposed to high light (HL) and methyl viologen (MV). The chlAPX knockdown plants were affected more severely than the non-transformed (NT) plants in photochemical activity and CO_2 assimilation in the presence of MV, suggesting that these enzymes are important for PSI and its associated components. Although MV induced significant increases in pigment content in the knockdown plants, the increases were apparently not sufficient for protection. Treatment with HL also caused generalized damage in PSII in both types of plants. The knockdown and NT plants exhibited differences in photosynthetic parameters in response to light and intercellular CO_2 pressure under normal growth conditions. The knockdown plants overexpressed other antioxidant enzymes in response to the stresses. Our data suggest that a partial deficiency of chlAPX expression modulated the PSII activity and integrity, reflecting the overall photosynthetic process when rice plants are subjected to acute stress at the level of PSI. However, under normal growth conditions, the knockdown plants exhibit normal phenotypes, biochemical and physiological responses.

INTRODUCTION

Ascorbate peroxidases (APXs) are heme-binding enzymes that reduce hydrogen peroxide (H_2O_2) to water using ascorbate (AsA) as an electron donor (Asada, 1999). In higher plants, APX isoforms are encoded by a small gene family, with different APX isoforms being targeted to distinct subcellular compartments: mitochondria (miAPX), cytosol (cAPX), peroxisome/glyoxysomes (mAPX), thylakoid membranes (tAPX) and stroma (sAPX) in chloroplasts (Teixeira et al., 2006). The APX isoenzymes, especially the chloroplastic APXs (chlAPXs), are unstable in low concentrations of ascorbate (AsA) and in the presence of H_2O_2 (Shigeoka et al., 2002; Ishikawa and Shigeoka, 2008). Decreases in AsA levels and inactivation of APXs in chloroplasts have been considered to be limiting

factors in the efficiency of the water-water cycle and photo-oxidative protection under stress conditions (Ishikawa and Shigeoka, 2008). The coordinate action of this cycle is essential to dissipate excess energy from PSII and avoid disturbances in photosynthesis (Asada, 1999).

The water-water cycle directly involves the chloroplast superoxide dismutase (Cu-ZnSOD isoform), which is attached to the PSI complex of the thylakoid membrane and physically near tAPX (Asada, 2006). tAPX operates in association with SOD exerting the control under photo-oxidative stress (Asada, 1999). Thus, under acute photo-oxidative stress generate by HL and MV the water-water cycle generates great amounts of superoxide and especially H_2O_2 (Ishikawa and Shigeoka, 2008). These ROS may be eliminated by other Cu-ZnSOD isoforms and sAPXs localized in the stroma in association with other enzymes in the ascorbate-glutathione cycle (Gill and Tuteja, 2010). Thus, both chloroplast APXs protect the photochemistry machinery against photo-oxidative damage and photoinhibition by consuming excess electrons from PSII (Kangasjarvi et al., 2008).

Frequently, under abiotic stress, CO_2 assimilation, which represents the most important electron sink from photosystems, is decreased (Chaves et al., 2009). In this situation, the thylakoid membrane is over-reduced by a strong “electron pressure” (Osmond and Förster, 2006). Chloroplasts have several energy dissipation mechanisms to cope with electron excess in the photosynthetic electron transport (PET) chain, such as the xanthophyll cycle that is associated with the thylakoid pH gradient and involved with heat dissipation by non-photochemical quenching (NPQ), transient photoinhibition and repair of PSII by synthesis *de novo* of the D1 protein and photorespiration (Demmig-Adams et al., 2006; Yin et al., 2010). To minimize the excess of electrons in the photosystems and to eliminate H_2O_2 produced by O_2 reduction and SOD activity, chloroplasts have other peroxidases such as peroxiredoxins and glutathione peroxidase (GPX) isoforms, which are able to scavenge H_2O_2 and other hydroperoxides (Pnueli et al., 2003; Mittler and Poulos, 2005). The effectiveness of these peroxidases in compensating for APX deficiency is still in question (Bonifacio et al., 2011). To

induce photo-damage, it is common to expose plants to intense light, which leads to the production of singlet oxygen ($^1O_2$) and the over-reduction at level of reactions center of PSII. In addition, under certain circumstances, the reducing side of PSII may form other ROS such as H_2O_2, superoxide and hydroxyl radicals from the reduction of molecular oxygen (Pospísil, 2009). This situation might lead to photo-damage and photoinhibition of PSII (Osmond and Förster, 2006).

Because the chlAPXs are immensely sensitive to H_2O_2, they are among the first molecules attacked by ROS in chloroplasts. Strangely, some mutant plants lacking or deficient in chlAPXs have not exhibited higher sensitivity to HL or MV compared with NT plants (Giacomelli et al., 2007; Kangasjarvi et al., 2008). However, other mutants over-expressing chlAPXs have shown higher resistance to abiotic stresses such as HL and MV (Pang et al., 2011). The role of chlAPXs in the photo-oxidative protection is still under debate, which highlights the following question: if the chlAPXs are essential for oxidative defense, why have a variety of studies shown contradictory results, often indicating little importance of these enzymes? A study with single and double mutants of *Arabidopsis* lacking cAPX and tAPX suggested that the current paradigm of the central pathways for ROS elimination involving APXs should be reviewed (Miller et al., 2007).

Recently, we demonstrated that rice plants deficient in both cytosolic APXs exhibit a compensatory mechanism employing other peroxidases, a mechanism that apparently does not occur in *Arabidopsis* (Bonifacio et al., 2011). Most of the studies of the effect of the lack or deficiency of chlAPXs have been carried out with *Arabidopsis*; however, several studies have shown that plants differ widely in their mechanisms of oxidative response, especially with regard to networks associated with perception, signaling, gene expression and protective physiological strategies (Pfannschmidt et al., 2009; Mullineaux and Baker, 2010). This variability is based in part on the large antioxidant metabolism redundancy and plasticity in higher plants (Miller et al., 2007). Thus, it is plausible that a plant such as rice, with a more complex genome than *Arabidopsis*, could support more complex gene networks and different metabolic and physiological mechanisms to cope with abiotic stress induced by oxidative damage (Bonifacio et al., 2011). Thus, a better

understanding of the role played by chlAPXs in monocot crop models such as rice could contribute to a more complete appreciation of the molecular, biochemical and physiological mechanisms that control plant tolerance to abiotic stress. We have previously shown that rice *OsAPX* genes are modulated by different stresses such as salt, drought and exogenous H_2O_2 (Teixeira et al., 2006; Rosa et al., 2010). We also studied the functional role of the cytosolic APXs and documented the importance of these enzymes to the ROS response (Rosa et al., 2010; Bonifacio et al., 2011). We demonstrated that, in contrast to *Arabidopsis,* rice plants silenced for both cytosolic APXs exhibited a compensatory mechanism involving the expression and activity of other peroxidases, especially isoforms of catalase (CAT) and GPX.

In the current study, we investigated *in vivo* subcellular localization of three soluble putative chloroplast isoforms of OsAPX. Next, we generated rice plants with knockdowns in both chloroplastic ascorbate peroxidases (*OsAPX7* and *OsAPX8*), and using an integrated approach involving gene expression, proteomics, enzymatic activities and a deep photosynthesis study, we have assessed the role of chlAPXs in the oxidative defense and the regulation of photosynthesis of rice plants exposed to acute stress conditions induced by high light and methyl viologen. Our data revealed that under acute stress conditions imposed by MV but not by HL, the chlAPXs were crucial to oxidative protection at the level of the water-water cycle. The intense disturbances caused in PSI triggered immense changes in PSII, such as photodamage, photoinhibition and inhibition of the mechanism of dissipation of excess energy as heat by NPQ. The effect was possibly due to the inhibition of the xanthophyll cycle and disruption of the pH gradient between the lumen and stroma, especially in the silenced plants. These alterations were associated with the blocking in the photosynthetic electron transport chain due to over reduction of the plastoquinone (PQ) pool. As a consequence of deficiencies in chlAPXs, knockdown plants suffered more oxidative damage and less CO_2 assimilation than the controls.

The deficiency of chlAPXs apparently was not essential for protection against the photodamage and photoinhibition triggered in PSII by high light.

Knockdown and NT plants exhibit differences in some important photosynthetic characteristics in response to light intensity and intercellular CO_2 pressure under normal growth conditions. The role of rice chloroplastic APXs in the regulation, integrity and activity of photosystems I and II during CO_2 assimilation was examined. In addition, the involvement of SOD and APX as acceptors of excess electrons in the thylakoid and stroma, oxidative protection and photosynthesis regulation under chlAPX deficiency was also discussed.

RESULTS

Subcellular Location of APX Proteins Revealed Dual Targeting to Chloroplasts and Mitochondria

Previously, we identified eight OsAPX proteins in the rice genome. Through *in silico* analysis, OsAPX5, OsAPX6, OsAPX7 and OsAPX8 proteins were characterized as putative chloroplastic isoforms (Teixeira et al., 2004). Next, we experimentally analyzed the subcellular location of the rice OsAPX6 isoform using a GFP-fusion protein in BY-2 tobacco cells. In contrast to the initial prediction, OsAPX6 was found in the mitochondria of the BY-2 cells (Teixeira et al., 2006). This result prompted us to investigate the localization of other soluble putative chloroplast isoforms. In the present work, to investigate the *in vivo* subcellular localization of OsAPX5, OsAPX6 and OsAPX7 proteins in rice, we constructed translational fusions of OsAPX5, OsAPX6 and OsAPX7 with the YFP gene driven by the CaMV 35S promoter. Transient expression was performed in rice protoplasts and analyzed with a confocal laser-scanning microscope. Confocal analysis of protoplasts expressing the 35S-OsAPX5::YFP, 35S-OsAPX6::YFP and 35S-OsAPX7::YFP fusions revealed that YFP fluorescence localized in mitochondria/chloroplasts (Fig. 1A-F), mitochondria (data not shown) and chloroplasts (Fig. 1G-I), respectively. Tetramethylrhodamine ethyl ester perchlorate (TMRE), a mitochondria-specific fluorescent marker, was used in rice protoplasts.

These results indicate that OsAPX5 is a dually localized protein, targeted to mitochondria and to chloroplasts (Fig. 1A-F). The YFP fluorescence of OsAPX5 was either localized in mitochondria or chloroplasts in different cells but never to both in the same cell. The subcellular location of the OsAPX6 protein was confirmed in mitochondria (data not shown) as previously demonstrated in BY-2 cells (Teixeira et al., 2006). The OsAPX7 protein was located in chloroplasts (Fig. 1G-I). The positive control for the transformations was the 35S::GFP (Green Fluorescent Protein) (Fig. 1J), and the negative control was the empty vector (Fig. 1K and L).

Doubled Silenced *OsAPX7*/*OsAPX8* Plants Display Decreased APX Activity in the Chloroplast Fraction and Slightly Altered Antioxidant Responses

To determine the functional role of the chloroplastic ascorbate peroxidase genes, both *OsAPX7* (stromal) and *OsAPX8* (thylakoid) were silenced using inverse repeat (IR) constructs that direct transcription of dsRNA (hairpin). Rice calli were transformed with the RNAi*Os*APX7/8 construct, and transgenic lines carrying the hairpin construct were recovered (Apx7/8s plants). The transgenic plants did not show phenotypic changes when compared with control plants grown under normal conditions (Fig. 2A). However, the RT-qPCR analysis of T0 plants showed that the mRNA levels of the chloroplastic *OsAPX* genes were reduced to 40% for *OsAPX7* and to 60% for *OsAPX8* in transgenic plants compared with the non-transformed (NT) plants (Fig. 2B).

The T1 generation of the Apx7/8s-2 line was used as the representative line for further experiments because its oxidative characterization by RT-qPCR and its enzymatic activities were similar to the other two lines. To investigate whether the other *OsAPX* genes could be modulated differently in the transgenic plants, we analyzed by RT-qPCR the transcript levels of *OsAPX1*, *OsAPX2*, *OsAPX3*, *OsAPX4*, *OsAPX5*, *OsAPX6*, *OsAPX7* and *OsAPX8* in Apx7/8s-2 plants compared to NT rice. *OsAPX7* and *OsAPX8* were the only genes showing statistically

significant reduction of expression in the T1 generation compared to NT plants (Fig. 2C).

These results indicate that in these plants, the expression of the chloroplastic *OsAPX* genes has been specifically reduced. As a consequence of the gene silencing, in both T0 and T1 plants, the APX activity in the chloroplastic fraction decreased by 50% (Fig. 3A and 12A), while in the cytosol fraction the APX activity did not change (Fig. 3B and 12B) compared to NT plants.

The CAT activity did not change significantly, while SOD activity showed a significant decrease in transgenic Apx7/8s plants compared to NT plants (Fig. 3C and D). The H_2O_2 steady-state level increased significantly in transgenic plants in comparison with NT plants (Fig. 3E), while TBARS content, an indicator of lipid oxidation did not show any alterations in transgenic Apx7/8s plants when compared to the NT plants (Fig. 3F). These results indicate that although Apx7/8s plants have lower chloroplastic APX activity and higher levels of H_2O_2, they did not display important alterations in their the redox homeostasis, despite the fact that the total SOD activity was 28% lower in Apx7/8s knockdown plants.

Proteomic Analysis of Apx7/8s Knockdown Plants Grown Under Normal Conditions Reveals Changes in Photosynthesis-Related Proteins

Proteomic analyses were performed to identify differentially expressed proteins in the rice *OsAPX* knockdown plants (Apx7/8s-2 line) grown under normal conditions. Several spots showed differential expression between silenced and NT plants, but only spots with a coefficient of variance less than 10% in a set of three replicates were selected for mass spectrometry analyses. Among all of the protein spots detected, only 19 proteins were significantly downregulated or upregulated in Apx7/8s plants compared with NT plants. A set of proteins that did not show significantly different expression (up or down) compared to NT plants were also analyzed but not included in Table 1. The 19 identified proteins were classified into six groups according to their metabolic function: (1) photosynthetic process, (2) response to oxidative stress, (3) amino acid metabolism, (4) phosphate

metabolism, (5) protein metabolic process, and (6) RNA degradation (Tab. 1). The largest protein group (48%) was associated with photosynthetic process, followed by proteins related to oxidative stress (26%) and amino acid metabolism (11%). The other groups represented 5% each (Fig. 4). These results showed that silencing chloroplastic *OsAPX* genes primarily affected the expression levels of proteins involved with the photosynthetic process and oxidative metabolism under normal growth conditions.

Apx7/8s Knockdown Plants Exhibited Alterations in Photosynthetic Pigments, Photochemical Activity and CO_2 Assimilation in Response to Abiotic Stresses

To verify the effect of the double *OsAPX7* and *OsAPX8* knockdown on responses to photoinhibition, photodamage and photooxidative stress, Apx7/8s plants were exposed to high light (HL) stress by a continuous exposure to a photosynthetic photon flux density (PPFD) of 2,000 μmol m^{-2} s^{-1} for 24 h or to 50 μM methyl viologen (MV) for 24 h. The redox-cycling agent MV is widely used as a source of superoxide radicals. No difference in the morphological phenotype, such as visible symptoms of injury, was observed between the knockdown and NT plants.

We evaluated the changes in the concentrations of pigments involved with photosynthesis and antioxidant protection (chlorophyll a and b, carotenoids and anthocyanins). Under normal growth conditions, Apx7/8s mutants did not exhibit changes in pigment content compared with NT plants (Fig. 5). The total chlorophyll content increased only in NT plants under HL stress, while the chlorophyll content decreased in both silenced and NT plants treated with MV compared with the respective controls, although the silenced plants displayed higher chlorophyll content (Fig. 5A). The chlorophyll a/b ratios followed the same trend as the total chlorophyll content (data not shown). Anthocyanins increased in both Apx7/8s and NT plants subjected to HL and MV stresses; however, the increase was more pronounced in the Apx7/8s plants (Fig. 5B). Carotenoids markedly decreased in

both stressed Apx7/8s and NT plants, but the effect of MV on the carotenoids was greater in NT plants (Fig. 5C). The fresh mass of shoots in the Apx7/8s and NT plants subjected to abiotic stress conditions was determined, but no difference was observed between the Apx7/8s and NT plants (data not shown).

To verify the integrity and functionality of the PSII, several parameters associated with chlorophyll a fluorescence were measured. In plants grown under normal conditions (control) the Fv/Fm was similar in both genotypes (Fig. 6A). This parameter is associated with the integrity of PSII and corresponds to the fraction of absorbed photons that are used in the photochemistry system for a dark-adapted leaf. Both HL and MV decreased Fv/Fm, but this effect was more accentuated in transgenic plants treated with MV (Fig. 6A). A significant decrease in Fv/Fm is commonly interpreted as an indicator of photoinhibition (reversible or irreversible) in the PSII. The ΔF/Fm' was also similar in both types of plant under control conditions. This photochemical parameter is associated with PSII efficiency, which represents the fraction of absorbed photons that are used in photochemistry for a light-adapted leaf. This parameter was drastically decreased in both types of plant exposed to HL stress. However, in the presence of MV only the knockdown plants were markedly affected (Fig. 6B). The ETR parameter, which represents the electron fraction effectively driving the reduction of $NADP^+$, did not change in response to *OsAPX7* and *OsAPX8* silencing under control conditions. The effects on ETR were similar to those observed for the ΔF/Fm'. Exposure to HL strongly decreased the ETR in both genotypes, while the effects of MV were more pronounced in the knockdown lines than in the NT plants (Fig. 6C).

The EXC, which corresponds to the energy fraction that cannot be used to drive the photochemical activity, was also not changed by the knockdown of *OsAPX7* and *OsAPX8* in control conditions. In contrast to the observations for ΔF/Fm' and ETR, the energy excess (EXC) was drastically increased by HL in both genotypes. Treatment with MV increased the EXC more in the knockdown plants than in the NT plants (Fig. 6D). Overall, in the Apx7/8s plants growing under normal conditions the photochemical activity and integrity were not altered, but when exposed to MV, they suffered more intense damage to the PSII compared

with NT plants. Similar to ΔF/Fm' and ETR, the photochemical quenching (qP) in HL stress decreased in both plants, but in the presence of MV only the silenced plants were clearly affected (Fig. 6E). The qP represents the fraction of photons absorbed by PSII that is driven to photochemical events or activity. The NPQ was similar in both genotypes under normal growth conditions (Fig. 6F). This parameter indicates the energy fraction dissipated as heat, which is associated with plant protection mechanisms developed to avoid over-energization of the thylakoid membranes. However, HL greatly increased NPQ while MV caused a strong decrease, especially in the silenced plants. These results reinforced the conclusion that MV has different effects on the two genotypes, with the silenced plants being much more sensitive than the NT plants.

Similar to the PSII activity, the gas exchange parameters associated with photosynthesis (P_N – net CO_2 assimilation, gs – stomatal conductance, Ci – intercellular concentration of CO_2 and E – transpiration) were significantly affected in Apx7/8s knockdown and NT plants exposed to HL and MV, especially plants exposed to MV. However, these parameters were similar among knockdown and NT untreated control plants (Fig. 7). Net CO_2 assimilation was strongly affected by HL and MV, but knockdown plants were more severely affected than NT plants (Fig. 7A). This trend was similar to that of stomatal conductance (Fig. 7B), while MV induced an increase in Ci only in the knockdown plants (Fig. 7C). In general, transpiration followed the same trend as net CO_2 assimilation and stomatal conductance in all treatments (Fig. 7D). Comparatively, MV was more dangerous than HL; moreover, the oxidative-stress modulator was more hazardous to transgenic plants than to NT plants.

In summary, under normal growth conditions, the photosynthetic pigments, photochemical activity and gas exchange of the Apx7/8s knockdown plants were similar those of the NT plants. However, the knockdown plants were more sensitive to MV-induced oxidative stress than the NT plants while HL induced similar levels of photodamage in the two genotypes. The exception was CO_2 assimilation, which decreased more in transgenic than in NT plants. Thus, in plants deficient in both chlAPXs compared to NT plants MV caused more severe

photochemical problems in the photosynthetic electron transport (PET) chain and CO_2 assimilation, such as photoinhibition, decreased PSII efficiency, impairment in electron transport rate and deficiencies in the dissipation of excess energy as heat (decreased NPQ).

The Knockdown of *OsAPX7* and *OsAPX8* Altered Potential Parameters Associated With Photosynthetic Efficiency

The Apx7/8s knockdown and NT plants were compared in terms of potential photosynthetic efficiency under optimum conditions of temperature, light and intercellular CO_2 concentrations. Initially, P_N - Ci fitting curve (photosynthesis depending of the intercellular concentration of CO_2) and P_N – PPFD fitting curve (photosynthesis depending of light intensity) (Fig. 8A and B) were performed, and some photosynthetic parameters associated with CO_2 photosynthetic efficiency were calculated by the mathematical model developed by Sharkey et al. (2007). The P_N - Ci curve showed two distinct phases, a linear phase from 0 to approximately 50 Pa of Ci (which is associated with the maximum Rubisco carboxylation rate - Vcmax) that indicates the rate of Rubisco turnover under optimum conditions, and a non-linear phase approaching saturation from approximately 40 to 80 Pa of Ci, which is associated with the rate of maximum photosynthetic electron transport (Jmax) that represents the electron flux based on the NADPH requirement for the Calvin cycle.

In addition, the model permits the estimation of other parameters associated with photosynthetic efficiency, such as photorespiration (Pr) as shown in Table 2. The Apx7/8s plants showed both Vcmax and Jmax 18% and photorespiration 30% higher than NT plants (Tab. 2). These results indicate that transgenic plants under optimum conditions potentially perform better in terms of Rubisco activity (turnover) and electron transport to NADPH formation. In addition, the P_N max in CO_2-saturated air was 20% higher in transformed plants. Under these optimum conditions, transgenic plants were more able to dissipate excess energy from the PET chain by photorespiration, as shown by higher light CO_2

release (light respiration, Rd), a measure of photorespiration + respiration (37%), Pr (photorespiration, 30%) and an approximately 31% higher CO_2-compensation point (Tab. 2). To compare the efficiency of light use, the P_N - PPFD curve was determined with the photosynthetic photon flux density changing from 0 to 2,000 µmol m^{-2} s^{-1} (Tab. 2). The NT plants showed maximum photosynthesis 27% higher than the Apx7/8s mutants with P_N saturation in both lines beginning at approximately 1,250 µmol m^{-2} s^{-1}. In addition, the Apx7/8s plants showed lower P_N in light intensity above 1,000 µmol m^{-2} s^{-1} than NT plants. The quantum efficiency (α), a parameter associated with conversion of one photon per assimilate-CO_2, was also lower in silenced plants (by 13%). These results reinforce the conclusion that knockdown plants were probably less efficient in converting light into assimilated carbon even if the other parameters such as Rn (dark respiration), θ (convexity of light curve) and the light compensation point did not show statistically significant differences among genotypes (Tab. 2).

The Expression of *OsAPX* and *OsGPx* is Altered in Response to Abiotic Stress in the Rice Plants Deficient in Chloroplastic *APX* Genes

To address the effect of silencing the chloroplastic *OsAPX* genes on the overall antioxidant cellular responses, we measured the effect of HL and MV abiotic stresses on the transcript levels of the following genes: *OsAPX1* and *OsAPX2* (cytosolic), *OsAPX3* and *OsAPX4* (peroxisomal), *OsAPX5* (mitochondrial/chloroplastic), *OsAPX6* (mitochondrial), *OsAPX7* and *OsAPX8* (chloroplastic) and *OsGPx4* (chloroplastic glutathione peroxidase 4). The normalization was performed with appropriate control plants. In the NT plants treated with HL (2,000 µmol m^{-2} s^{-1} for 24 h), the *OsAPX1* and *OsAPX6* genes were significantly upregulated. Interestingly, only the *OsAPX8* gene was downregulated (Fig. 9A). Similarly, HL treatment strongly increased the expression of these same genes (*OsAPX1 and OsAPX6*) and reduced the expression of the *OsAPX8* gene in the Apx7/8s knockdown plants. However, these plants also responded by increasing the expression of *OsAPX7* in response to the stress (Fig. 9B). In NT plants, the transcript accumulation levels were significantly increased

for *OsAPX1* after 24 h of 50 µm MV treatment, while *OsAPX8* expression was significantly reduced (Fig. 9C).

The Apx7/8s knockdown plants treated with MV showed significant induction of the cytosolic *OsAPX1* and chloroplastic *OsAPX7* genes (Fig. 9D). Reduced transcript levels were observed only for *OsAPX8* (Fig. 9D). The transcript levels of the *OsGPx4* gene were significantly increased in both knockdown and NT plants exposed to HL treatment (Fig. 10A). Interestingly, in plants treated with MV, the transcript accumulation levels of the *OsGPx4* gene were strongly enhanced only in Apx7/8s plants (Fig.10B). These results indicate that knockdown plants exposed to HL and MV stress showed different levels of transcripts involved with oxidative protection than NT plants submitted to the same stress conditions.

Apx7/8s Plants Exposed to High Light and MV Stress Show Different Alterations in the Antioxidant Response Compared to NT Plants

To assess whether a deficiency in the chloroplastic APXs could change the antioxidant response under abiotic stress, we evaluated the changes in the concentrations of some antioxidants and enzymes involved in antioxidant protection in addition to some oxidative stress markers. The Apx7/8s plants exhibit higher levels of H_2O_2 in control conditions and after exposure to HL than the NT plants (Fig. 11A). When treated with HL, knockdown plants showed higher increases in the reduced ascorbate content than the NT plants (Fig. 11B). However, in the knockdown plants this increase was associated with a higher redox state of this antioxidant (data not shown). When treated with HL Apx7/8s and NT plants showed similar and prominent increases in membrane damage, as indicated by electrolyte leakage (Fig. 11C). Under normal growth conditions Apx7/8s and NT plants showed similar TBARS contents, an indicator of lipid peroxidation. However, MV and HL induced very large increases in lipid peroxidation in both knockdown and NT plants, but the MV-induced increase was greater in Apx7/8s plants than in the NT plants (Fig. 11D). The H_2O_2 concentration was higher in knockdown plants treated with MV than in NT plants (Fig. 11A).

However, the levels of reduced ascorbate decreased similarly in both Apx7/8s and NT plants (Fig. 11B). The higher H_2O_2 accumulation paralleled higher membrane damage and lipid peroxidation in the knockdown plants (Fig. 11A, 11C and 11D).

Altogether, these data indicate that the plants in which the chloroplastic *OsAPX* genes were knocked down showed slight changes in the antioxidant responses under normal growth conditions but distinct changes in response to abiotic stresses. Overall, the knockdown plants were more sensitive than the NT plants to MV-induced oxidative stress, as indicated by higher levels of membrane damage, lipid peroxidation and hydrogen peroxide. The simultaneous knockdown of the *OsAPX7* and *OsAPX8* genes induced a decrease (50%) in the activity of chloroplast APX in non-stressed transgenic plants compared with the NT plants (Fig. 12A). Interestingly, the chloroplastic APX activity of silenced plants remained unchanged in response to HL and MV stresses compared with the control condition. In contrast, in NT plants, chloroplast APX activity was significantly increased, especially in response to HL (Fig. 12A). The APX activity in the cytosolic fraction after HL or MV stress increased slightly in both knockdown and NT plants (Fig. 12B), whereas CAT activity was marginally altered by HL, MV and silencing (Fig. 12C).

Silencing slightly decreased the total SOD activity (Fig. 3D), but HL caused strong decreases in SOD activity of both types of plants. Interestingly, MV triggered clear increases in SOD activity only in knockdown plants compared to the corresponding controls (Fig. 12D). The chloroplastic GPX activity in knockdown plants was greatly increased by both HL and MV treatments (Fig. 12E), whereas in the cytosol fraction the GPX activities in both types of plant and both treatments were slightly reduced; however, the knockdown control plants showed higher GPX activity than the control NT plants. Together, these results demonstrate that the simultaneous knockdown of the *OsAPX7* and *OsAPX8* genes changes antioxidant metabolism in transgenic plants exposed to oxidative stress and leads to alterations in the activity of important enzymes, such as SOD and GPX.

DISCUSSION

In rice, the eight members of the *OsAPX* gene family appear to play different roles during plant development and response to abiotic stress (Teixeira et al., 2006; Rosa et al., 2010). Among the eight rice APXs, four proteins were identified as putative chloroplastic isoforms (OsAPX5, OsAPX6, OsAPX7 and OsAPX8) (Teixeira et al., 2004). Using the expression of an OsAPx6-GFP protein fusion in BY-2 tobacco cells, we have previously demonstrated that in contrast to the initial prediction, OsAPX6 was found in mitochondria (Teixeira et al., 2006).

In the present study, we confirmed that in rice protoplasts OsAPX6 is a mitochondrial protein (data not shown) and that OsAPX7 has a chloroplastic localization (Fig. 1G-I). OsAPX5, in contrast, is a protein with dual localization, targeted to both chloroplasts and mitochondria (Fig. 1A-F). These results corroborate those obtained by Lazzarotto et al. (2011), who showed that APX-R, a protein functionally associated with APX that can interact with OsAPX5, is a dually localized protein, targeted to both chloroplasts and mitochondria in rice. Therefore, among the eight APX present in rice, only two proteins, APX7 and APX8, are specifically targeted to the chloroplasts. The production of plants deficient in different APX isoforms will permit us to understand the role of these enzymes in the antioxidant mechanisms in different subcellular compartments. Here, the role of chloroplast isoforms was assessed using an RNAi strategy. In spite of all our efforts to produce knockdown plants for the *OsAPX5* gene, the calli expressing *OsAPX5*-RNAi never regenerated into plants. In contrast, *OsAPX7* and *OsAPX8* knockdown plants were viable and fertile.

Deficient Plants in *OsAPX7* and *OsAPX8* do not Suffer Oxidative Damage When Growing Under Control Conditions

Some investigators have reported that the lack or deficiency of chloroplastic APXs triggers alterations in plant phenotypes and oxidative responses even under normal growth conditions (Giacomelli et al., 2007; Kangasjarvi et al., 2008; Pang et al., 2011). However, rice plants knockdown in

both chlAPXs growing under normal conditions presented a normal morphological phenotype (Fig. 2A) and did not exhibit symptoms of oxidative damage, as indicated by membrane damage and lipid peroxidation assays, in spite of having higher H_2O_2 concentrations compared to NT plants (Fig. 11A, C and D). The increased H_2O_2 might be a result of lower APX activity in chloroplasts due to silencing of the chlAPXs (Fig. 3A and 12A). This alteration can be an adaptive advantage because H_2O_2 is a powerful signal in chloroplasts and in all plant cells (Osmond and Förster, 2006).

Hydrogen peroxide signaling is associated with several physiological processes involving growth and development (Athanasiou et al., 2010), the expression of several genes involved with photosynthesis (Ivanov et al., 2008), plant defenses against biotic and abiotic stresses (Asada, 2006) and several other processes and mechanisms. Our results agree with those observed using single mutants lacking either tAPX or sAPX, which showed no phenotype changes (Maruta et al., 2010). In addition, *Arabidopsis* plants with a 50% reduction in tAPX activity are indistinguishable from control plants under normal growth conditions (Tarantino et al., 2005). In contrast, Danna et al. (2003) observed that a reduction of 40% in the tAPX activity in wheat plants led to a decrease in growth and seed production. Comparing different studies carried out by different investigators is not a simple task and must be performed with caution because the analysis of mutant phenotypes for antioxidant genes is very dependent on the growth conditions.

Proteomic Analysis of Apx7/8 Knockdown Plants Shows Upregulation of Photosynthesis-Related Proteins

The proteomic analyses revealed several metabolic pathways affected by chloroplastic APX silencing in rice plants (Fig. 4). The major pathways and/or processes affected in the knockdown plants were photosynthesis, antioxidant metabolism, amino acid metabolism linked to photorespiration and other proteins involved in phosphate metabolism, metabolic protein processing and RNA degradation. In the photosynthetic pathway, the majority of the proteins were

upregulated in knockdown plants. A similar trend was observed in other pathways (Tab. 1) indicating that the knockdown of chloroplastic *APX* genes in rice affects the abundance of proteins involved in different metabolic mechanisms, especially those associated with photosynthesis. Possibly, these changes triggered by chlAPX silencing might be associated with the high level of H_2O_2 in silenced plants. Indeed, H_2O_2 is a powerful signal for photosynthesis reactions modulating the expression of several chloroplastic genes (Asada, 2006; Goh et al., 2011). In addition, H_2O_2 also can act as a strong inhibitor of photosynthetic reactions. Even at low concentrations, it can inhibit CO_2 fixation by 50% because of the oxidation of the thiol-modulated enzymes of the Calvin cycle (Foyer and Shigeoka, 2011). Among the proteins related to photosynthetic processes, the Rubisco activase small isoform precursor was upregulated in the Apx7/8 knockdown plants (Tab. 1).

However, the expression of the smaller and larger Rubisco subunits was not altered (data not shown). Rubisco activase plays a key role in Rubisco activity (Osmond and Förster, 2006). In addition, the expression of putative chloroplastic glyceraldehyde 3-phosphate dehydrogenase was upregulated in the knockdown plants. This enzyme is involved with the NAD(P)H-dependent reduction of glyceraldehyde 3-phosphate, which is a precursor of the glyceraldehyde 3-phosphate that might be involved in the ribulose 1,5-bisphosphate regeneration (Foyer et al., 2012). Putative ferredoxin-NADP(H) oxidoreductase, a key protein involved with electron transport from photosystems to the Calvin cycle, was also upregulated in silenced plants. Other important enzymes of the Calvin cycle, such as phosphoglycerate kinase, which is involved with Rubisco oxygenase activity (photorespiration), were upregulated in knockdown plants. Interestingly, this change was associated with upregulation of the putative glycine dehydrogenase and glycine cleavage systems, both involved with photorespiratory activity in mitochondria.

It is very important to note that the alterations revealed by the proteomic analysis are closely associated with the changes in the photosynthesis and photorespiration parameters measured “in vivo” by gas exchange (Tab. 2). In fact, under higher CO_2 concentrations and optimal light conditions (1000 μmol m^{-2} s^{-1}),

the silenced plants exhibit higher Rubisco maximum turnover rates, higher maximum electron transport rates to the Calvin cycle, higher photorespiration and higher maximum photosynthesis under saturating CO_2 conditions. Thus, the knockdown of chloroplastic *APX* genes in rice plants triggers marked changes in the expression of the photosynthetic genes, allowing these plants to increase CO_2 assimilation under high intercellular CO_2 concentrations. This observation implies that knockdown plants under optimum growth conditions could have lower photosynthesis under ambient CO_2 concentration because they have higher photorespiration. However, this potential condition of higher photorespiration apparently is not sufficient to avoid or to restrict the higher photochemical, photosynthesis and oxidative disturbances triggered by strong oxidative stress induced by MV.

Knockdown Plants Exhibit Similar PSII Activity and Less CO_2 Assimilation When Photodamage is Induced by High Light but are More Sensitive to MV-Induced Oxidative Stress

Under conditions of excess light, the production of ROS is accelerated in both PSI and PSII, but different ROS are produced by each photosystem (Goh et al., 2012). In PSII, the HL primarily induces the formation of singlet oxygen ($^1O_2$) mainly from the excited triplet state of chlorophyll (Asada, 2006). HL induces several oxidation-reduction alterations of the components of the PSII reaction centers causing over-oxidation of P680 that, together with $^1O_2$, can cause photodamage and photoinhibition of PSII (Osmond and Förster, 2006). In parallel, HL can also cause over reduction and protonation of the plastoquinone pool. In this chemical state, PQ_{red} do not function in the electron transport to PSI (Joliot and Johnson, 2011).

The photodamage and photoinhibition of PSII can be reversible or irreversible. Our results showed that HL strongly altered the PSII activity in both knockdown and NT plants (Fig. 6). Interestingly, although $\Delta F/Fm'$, ETR and qP decreased substantially, the HL-induced reduction in Fv/Fm apparently does not

indicate an irreversible photoinhibition in both genotype. Because the NPQ increased significantly (Fig. 6F), it is possible that this mechanism of dissipating excess energy as heat could help to avoid or minimize irreversible photoinhibition in rice plants. In contrast to the response exhibited by PSII, the decrease on the CO_2 assimilation was stronger in knockdown plants than in NT plants (Fig. 7). These strong effects of HL on CO_2 assimilation suggests that biochemical reactions of the Calvin cycle could have been affected because the stomatal conductance was similarly decreased in both knockdown and NT plants (Osmond and Förster, 2006). This situation (low CO_2 assimilation) could be a condition contributing to the low electron transport rate from PSII to PSI and to $NADP^+$, which would limit the concentrations of NADPH and ATP available to the Calvin cycle.

Interestingly, the photosynthetic pigments were less affected by the silencing in both HL and MV stress conditions. However, knockdown and NT plants exhibit slightly different responses. In general, MV strongly decreased the chlorophyll and carotenoid contents in both genotypes (Fig. 5A and C). Moreover, the concentrations of chlorophylls, anthocyanins and carotenoids increased in response to MV only in knockdown plants (Fig. 5B), while HL did not alter chlorophyll and carotenoid contents in either genotype (Fig. 5A and C).

Anthocyanins are important pigments involved in excess light dissipation and oxidative protection in chloroplasts, especially under stress conditions (Miller et al., 2007). The increased concentrations of these pigments in the knockdown plants under MV or HL stress conditions could have contributed, at least partially, to compensate for the deficiency of chlAPXs in terms of dissipating the excess energy in the photosystems, especially PSI. Methyl viologen is an extremely reactive compound capable of interrupting electron transport to ferredoxin. In the presence of an electron source, such as the photosynthetic electron transport (PET) chain near to PSI, MV transfers electrons to O_2 instead to ferredoxin, producing a huge amount of superoxide radicals and indirectly other ROS around to water-water cycle and PSI. A large amount of H_2O_2 is rapidly formed by SOD activity, and thylakoid APX is inactivated by peroxide excess (Asada, 2006). In a

short time, the water-water cycle collapses, and the excess superoxide and hydrogen peroxide is scavenged by the ascorbate-glutathione cycle. However, this cycle is not enough to eliminate the high concentration of those ROS. Under these conditions, progressively all chloroplast components are attacked by ROS (PSII and PSI, thylakoid membranes, Calvin cycle components, especially Rubisco (Osmond and Förster, 2006).

In the present study, MV caused serious disturbances in PSII activity and integrity and possibly also in PSI. Knockdown plants were more affected by MV than NT plants. The MV caused photoinhibition (indicated by the 0.82 – 0.45 change in Fv/Fm) and drastic decreases in PSII activity affecting all of the measured parameters (Fig. 6). It is important to note that NPQ greatly decreased, especially in the knockdown plants (Fig. 6F), indicating that processes associated with the dissipation of excess energy were severely affected. Indeed, the inhibition of the xanthophyll cycle was probably associated with the rupture in the pH gradient among lumen and stroma (Joliot and Johnson, 2011). As a consequence of the restriction in the electron transport from PSII to $NADP^+$ and possibly to ATP production, the CO_2 assimilation was substantially decreased, especially in the silenced plants (Fig. 7A).

As expected, our results emphasize that both chlAPXs are important for the integrity and function of the PSI and water-water cycle. Of course, the disturbances in PSI are immediately transferred to PSII and the Calvin cycle reactions (Asada, 2006). However, our data clearly show, for the first time, that the two chlAPXs are essential to the whole photosynthetic process affecting both photochemical and biochemical phases. However, our data suggest that the links between PSII and water-water cycle are very close and that, as has been recently suggested (Asada, 2006; Joliot and Johnson, 2011), this cycle is crucial not only for PSI or oxidative protection but also for PSII protection. In this context, chlAPXs are also crucial for the protection of the entire photosystem.

MV Induced More Oxidative Damage and Enzymatic Alterations in Silenced Plants Than in NT Plants, While HL Affected Both Silenced and NT Plants

As a consequence of the intense photodamage and photoinhibition of PSII and the serious disturbances in the chloroplastic PET chain caused by MV, especially in the chlAPX deficient plants, these plants suffered more oxidative damage than NT plants. Indeed, all oxidative damage indicators studied (H_2O_2, lipid peroxidation and electrolyte leakage) were higher in knockdown plants submitted to MV. Despite the capacity of MV to induce the activities of SOD and GPX in the knockdown plants (Fig. 12D and E), apparently this response was not sufficient to provide effective protection against ROS excess. Similar results were previously obtained in rice exposed to other abiotic stress factors such as salt and heat (Bonifacio et al., 2011).

Interestingly, despite the acute MV-induced stress, none of the activities of the antioxidant enzymes analyzed were decreased. These results suggest that the expression, turnover and structural integrity of these enzymes were not affected by MV-induced oxidative stress. In contrast, HL greatly decreased SOD activity in both knockdown and NT plants. Similar to MV, HL caused significant oxidative stress, but the ascorbate and antioxidant enzyme responses were different in both genotypes. In both genotypes, HL decreased SOD activity and, in contrast, strongly stimulated chlGPX activity only in the knockdown plants, whereas MV strongly increased the activities of both enzymes (Fig. 12 D and E). It is important to emphasize that rice plants are relatively sensitive to HL and MV (Bonifacio et al., 2011).

It is remarkable that the photochemical, CO_2 assimilation and enzymatic activities were very similar when the responses to MV and HL were compared in both genotypes. In fact, MV induced more severe damage (photodamage, photoinhibition, oxidative stress, photochemical and photosynthesis disturbances) in the knockdown plants, while HL caused similar levels of damage in the two genotypes. Our data strongly suggest that an approximate 60% deficiency in the expression of the two chlAPX together with a similar downregulation of APX activity in the chloroplast fraction triggered strong alterations in the overall

photosynthetic process. However, knockdown plants were more affected by MV, but they were not affected differently by HL (with the exception of CO_2 assimilation, which was decreased) compared to NT plants, indicating that the chlAPX deficiency affected some processes associated with PSI.

Because thylakoid APX participates with SOD in the water-water cycle in the inside of thylakoid membranes near PSI, a more effective role of APX in PSI in knockdown plants is, indeed, expected. As the H_2O_2 steady-state concentration is increased in silenced plants, it is plausible that ROS could be a signal involved in the oxidative network compensating for the deficiency of chlAPXs, as demonstrated recently for cytosolic APXs (Bonifacio et al., 2011). This compensation could confer a normal phenotype and a basal oxidative protection on transgenic rice plants grown under normal conditions. However, under artificial and acute oxidative stress, chloroplasts of the Apx7/8s knockdown plants suffer significant oxidative stress. In double silenced and NT plants, the expression of the *OsAPX* gene family and *OsGPx4,* an important chloroplastic antioxidant gene, were evaluated under HL and MV stresses. In these plants, HL and MV caused a strong modulation of *OsAPX1*, *OsAPX6*, *OsAPX7* and *OsGPx4* transcripts levels (Fig. 9 and 10), indicating the importance of these isoforms in protecting plant cells from abiotic stress.

Studies with *Arabidopsis* mutants deficient in chloroplastic APXs have showed that the absence of tAPX and/or sAPX induced no drastic compensatory increases in the steady-state levels of other APX isoforms. However, under high light stress, the absence of two chlAPXs induced an apparent compensatory increase in the steady-state level of 2-Cys PRX (2-cysteine peroxiredoxin) in chlAPX double-mutant plants (Kangasjarvi et al., 2008). Interestingly, the *OsAPX7* gene was also upregulated in double mutants submitted to abiotic stress. This upregulation suggests that in spite of the knockdown of this gene, it is still able to respond to the stress in an effort to compensate for its own post-transcriptional silencing triggered by the RNAi construct. However, the expression of *OsAPX7* is lower in the knockdown plants than it is in the NT plants. Remarkably, the *OsAPX8* gene was downregulated by abiotic stress (Fig. 9 and 10). The downregulation of

OsAPX8 in response to stresses such as salt (Teixeira et al., 2006; Yamane et al., 2010) and drought (Rosa et al., 2010) has already been reported. This reduction in the expression of *OsAPX8* might partially explain the good performance of the knockdown plants under stress because it could be advantageous for the plant to maintain low levels of *OsAPX8* mRNA during stressful situations.

CONCLUSIONS

Although many studies have demonstrated that the chlAPX enzymes might contribute significantly to the protection of chloroplasts from oxidation (Yabuta et al., 2002; Murgia et al., 2004; Miller et al., 2007; Kangasjarvi et al., 2008; Maruta et al., 2010), conflicting results have been reported (Miller et al., 2007). In general, the evaluation of the protective roles of the chlAPXs primarily utilizes approaches based on indirect measurements, especially of gene expression (transcript levels, microarray or proteomic analyses) and single gene knockout/knockdown transgenic plants. Moreover, most of these studies are of *Arabidopsis*. The approaches usually utilized to assess the functional role of chlAPXs and other antioxidative proteins have at least two basic limitations. First, the plant performance in terms of physiological responses, especially the photosynthesis regulation, is still poorly explored. For instance, when the oxidative protective role of chlAPXs is studied, generally properties such as photosystem activity, photosynthetic efficiency, biochemical reactions associated with antioxidant metabolism, and photosynthetic pigments are studied in a fragmented manner. Second, the studies carried out with *Arabidopsis* do not necessarily mimic a real condition of crop plants growing in a natural environment. Moreover, there are just a few studies of monocot species involving chlAPX proteins, especially studies of the molecular, physiological and biochemical characteristics of the proteins.

In the current study, employing a model crop plant, we use an integrated strategy involving genetic, proteomic, biochemical and physiological approaches, allowing us to assess the roles of chloroplastic APX enzymes under acute stress able to cause severe damage (photodamage, photoinhibition and blockage of

electron transport via PQ) to the PSII and the PSI. Our data support the conclusion that APX7 and APX8 are essential to both photosystems: initially PSI is exposed to acute oxidative stress, but the generalized oxidative disturbance caused, at least in part, by chlAPX deficiency, affects the PSII. Therefore, rice chlAPXs are essential to the whole photosynthetic process in rice plants under abiotic stress. However, a 60% deficiency of both APX transcripts, associated with a 50% decrease in APX activity in a chloroplast enriched fraction, does not impair the development of a normal plant under normal growth conditions.

MATERIALS AND METHODS

Construction of the plant vector and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsAPX7* (LOC_Os04g35520) and *OsAPX8* (LOC_Os02g34810*)* genes. The following primer pairs were used to amplify a 238-bp RNAi*OsAPX7/8* sequence: 5'-CACCCTCTAAAGCTTGTCCAAC-3' and 5'-TCAAGACCCATCCTGTAA-3'. PCR products were cloned into the Gateway vector pANDA, in which hairpin RNA is driven by the maize ubiquitin promoter and an intron placed upstream of the inverted repeats (Miki and Shimamoto, 2004). *Agrobacterium tumefaciens*-mediated transformation was performed as described previously (Upadhyaya et al., 2002).

The subcellular localization of the OsAPX5, OsAPX6 and OsAPX7 proteins was performed by transient expression of 35S-OSAPX5::YFP, 35S-OsAPX6::YFP and 35S-OsAPX7::YFP in rice protoplasts. The complete cDNAs of the OsAPXs were fused to the YFP coding sequence at its N-terminus and cloned into the pART7-HA-YFP plasmid (Galvan-Ampudia and Offringa, 2007) under control of the 35S promoter of CaMV. The amplified cDNAs were introduced into suitable plasmids using the Gateway technology. The resulting vectors were used for protoplast transformation.

Protoplast isolation was performed essentially as described (Chen et al., 2006), and protoplast transformation followed the methodology previously reported (Tao et al., 2002). Transformed protoplasts were incubated in the dark for 24–48 h at 27 °C prior to imaging. Fluorescence microscopy was performed with 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. Images were captured with a high-sensitivity photomultiplier tube detector.

Plant material and growth conditions

The rice plants with knockdowns of both *OsAPX7* and *OsAPX8* were obtained by *Agrobacterium tumefaciens*-mediated transformation of rice embryogenic calli (*Oryza sativa* L. ssp japonica cv. Nipponbare) induced from seeds and cultivated in NB medium (Upadhyaya et al., 2002) at 28°C in the dark. Rice seeds from the F1 generation of non-transformed (NT) and transgenic lines, in which the chloroplastic *OsAPX7* and *OsAPX8* genes (Apx7/8s) had been silenced, were germinated in MS medium (Sigma-Aldrich) supplemented with hygromycin under controlled conditions (150 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), 25°C, 80% relative humidity and a 12 h photoperiod). Two weeks after being sown, the rice seedlings were transferred to plastic 2-L pots (three seedlings per pot) filled with ¼ strength Hoagland-Arnon's nutritive solution (Hoagland and Arnon, 1950). The seedlings were grown for 2 months in a greenhouse (29 °C mean temperature, 68% mean relative humidity, average PPFD of 550 μmol m^{-2} s^{-1} and a 12-h photoperiod).

Methyl viologen (MV) and high light (HL) treatments

The 2-month-old transformed and NT plants were grown as described previously. For the MV treatment, a group of plants were transferred to a growth chamber at 27°C/24°C (day/night) and 70% humidity with a PPFD of 500 μmol m^{-2}

s^{-1}. The plants were then acclimated for 24 h. The MV was dissolved in 0.1% Triton-X-100 at 50 µM and sprayed in excess on the leaves of the plants until complete wetting was achieved. This procedure was repeated twice a day. The first symptoms of toxicity (brown spots) appeared on the leaves after 24 h of treatment. The control plants were sprayed with 0.1% Triton-X in the same way as the plants that underwent MV treatment. For the HL experiment, a group of 2-month-old transformed and NT plants were transferred to the same growth chamber. The plants were acclimated under low light intensity (200 µmol m^{-2} s^{-1}) for 24 h. For HL stress, the plants were continuously exposed to a PPFD of 2,000 µmol m^{-2} s^{-1} for 24 h. This light intensity was achieved with a combination of a high-pressure 400 W sodium vapor lamp (OSRAM PLANTASTAR®, Germany) and fluorescent 60 W lamps (Phillips®). Plants grown under a PPFD of 550 µmol m^{-2} s^{-1} were used as controls.

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with Trizol (Invitrogen). The cDNAs were obtained using the SuperscriptTMII reverse transcriptase system and a 24-polyTV primer (Invitrogen). After synthesis, cDNAs were diluted 10 to 100 times in sterile water for use in PCR reactions. Primers were designed to produce DNA fragments ranging from 180 to 250 bp. Subsequent PCR amplifications were performed using specific primers for *OsAPX1* to *OsAPX8* and *OsGPx4* genes: *OsAPX1*: (5'-CTCGAGCTACAAGGAGGCCCACCTCA-3') and (5'-GGTACCTCAGCCGCATTTCATACCAACACA-3'); *OsAPX2*: (5'-CTCGAGCCAAGTGACAAAGCCCTCAT-3') and (5'-GGTACCTCGAGAAGGCGCAAAATACAAATCG-3'); *OsAPX3*: (5'-GGTACCTCGAGCGAAAGATCAGGATTTGATGGT-3') and (5'-CTCGAGCCCAGCTCAGATAGCTTCTTGT-3'); *OsAPX4*: (5'-CTCGAGTGACAAGGCATTGTTGGAAG-3') and (5'-GGTACCTCGAGCAGCTGCAGCAACAGCTACC-3'); *OsAPX5*: (5'-CTCGAGAGGGCAATGTTGGACATCTG-3') and 5'-

GGTACCTCGAGGATCAAACTTTGCCCCAAGA-3'); *OsAPX6*: (5'-CTCGAGAGGGCAATCTTGGACATCAC-3') and (5'-GGTACCTCGAGGATCAAACTTTGCTCCGAGA-3'); *OsAPX7*: (5'-GAGCAATCTGGGTGCAAAAT-3') and (5'-GGTACCTCGAGGACTCGTGGTCAGGAAAAGC-3'); *OsAPX8*: (5'-CTCGAGGCTGCGAAATACTCCTACGG-3') and (5'-GGTACCTCGAGAGGAGGTCATCAGACCATCG-3'); *OsGPx4*: (5'-CTGTACATATGCCTTGCCTCA-3') and (5'-GTTACAGGGGCCAGATAAGC-3'). Primer pairs to amplify the *OsFdh3* gene, (5'-TTCCAATGCATTCAAAGCTG-3') and (5'-CAAAATCAGCTGGTGCTTCTC-3'), the *OsUbi* gene (5'-ACCACTTGCACCGCCACTACT-3') and (5'-ACGCCTAAGCCTGCTGGTT-3') and the *OsActina2* gene (5'-GGACGTACAACTGGTATCGTGTT-3') and (5'-GTTCAGCAGTGGTAGTGAAGGAG-3') were used as internal controls to normalize the amount of mRNA present in each sample. All of the reactions were repeated 4 times, and expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008). All of the RT-qPCR reactions were performed in an Applied Biosystems StepOne plus Real-time PCR system using SYBR-green intercalating-dye for fluorescence detection.

Gas exchange and photochemical parameters

The gas exchange measurements and the following photochemistry parameters associated with the efficiency of photosystem II were measured after 24 h of exposure to MV and HL: $\Delta F/F_m'$, actual quantum yield of photosystem II, F_v/F_m, potential quantum yield of photosystem II, and ETR, apparent electron transport rate. The relative excess energy at the photosystem II level was calculated as EXC = $[(F_v/F_m - \Delta F_v/F_m')/F_v/F_m]$. All of the parameters were measured with an Infrared Gas Analyzer coupled with a leaf chamber fluorometer (Li-6400-XT, LI-COR, Lincoln, USA) according to the manufacturer's instructions. ETR was calculated as ETR = ($\Delta F/F'_m$ x PPFD x 0.5 x 0.84), where 0.5 is the presumed fraction of the excitation energy distributed to PSII and 0.84 is the

assumed fraction of light absorbed by the leaf. EXC was calculated according to Bilger et al. (1995) as EXC = $[(F_v/F_m) – (\Delta F/F'_m)]/(F_v/F_m)$.

The photochemical quenching coefficient [qP = (Fm' – Fs)/(Fm' – Fo')] and the nonphotochemical quenching coefficient [NPQ = (Fm - Fm')/Fm'], where Fm and Fo are, respectively, maximum and minimum fluorescence of dark-adapted leaves; Fm' and Fs are, respectively, maximum and steady state fluorescence in the light-adapted state, and Fo' is minimum fluorescence after far-red illumination of the previously light exposed leaves. A saturating pulse of red light (0.8 s, 8000 µmol m^{-2} s^{-1}) was utilized.

Leaf gas exchange measurements were made using an Infra-red Gas Analyzer (Li-6400-XT, LI-COR Biosciences Inc., Lincoln, USA). Light was provided by a red/blue LED light source at photon irradiance of 1000 µmol m^{-2} s^{-1}. All leaf measurements were taken under ambient CO_2 conditions (380 ppm) at a constant leaf temperature of 28 °C and relative humidity of 78% and vapor-pressure deficit (VPD) 1,8. Gas exchange variables measured were net photosynthesis (P_N) and stomatal conductance (g_S), transpiration (E) and intercellular CO_2 concentration (CI).

Determination of photosynthetic efficiency parameters from P_N-Ci and P_N-PPFD fitting curve models

The measurements were performed in plants grown under natural conditions of temperature (29 °C), vapor-pressure deficit (1,8), relative humidity (68%) and PPFD (550 µmol m^{-2} s^{-1}) in a greenhouse. One fully expanded leaf was utilized for measurements. The P_N-PPFD and P_N-Ci curves were determined according to Lieth and Reynolds (1987) and Sharkey et al. (2007), respectively. Several parameters were calculated from those curves, including: maximum photosynthetic rate (P_{Nmax}), photosynthetic quantum efficiency (α), dark respiration (R_n), convexity of the curves that represent the estimate photosynthetic efficiency (θ), maximum Rubisco carboxylation rate (V_{cmax}), maximum rate of photosynthetic electron transport (J_{max}), triose phosphate use (TPU) light respiration (R_d), light-

and CO_2-compensation points. The photorespiratory rate (P_r) estimation was determined as described in Bagard et al. (2008) from the measurements of gas exchange (P_N and R_d) and chlorophyll *a* fluorescence-derived ETR parameter by the equation: $P_r = 1/12[ETR-4(A+R_d)]$.

Pigment determination

The total chlorophyll, chlorophyll *a* and *b* and carotenoid contents were determined after extraction in ethanol and were measured spectrophotometrically at 665 and 649 nm. The amount of pigment was calculated using the equations proposed by Lichtenthaler and Wellburn (1983). Anthocyanin was measured in according to Gitelson et al. (2001) after extraction in methanol-HCl 0.1% and calculated using the absorption coefficient of 30 mM^{-1} cm^{-1}.

Membrane damage and lipid peroxidation

Membrane damage or cellular viability was measured by the tissue K^+ leakage as described previously by (Cavalcanti et al., 2004) with minor modifications. Twenty leaf discs (1.0 cm in diameter) were placed in test tubes containing 20 mL deionized water. The flasks were incubated in a shaking water bath at 25 °C for 12 h, and the K^+ content was measured in the medium (L1) by a flame photometer (Micronal, Brazil). Then, the discs were boiled at 95 °C for 1 h, cooled to 25°C, and the K^+ content (L2) was measured again. The relative membrane damage (MD) was estimated using the formula: MD[%]=L1/L2×100. Lipid peroxidation was measured using thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak and Horst (1991), with minor modifications described by Rosa et al. (2010). The concentration of TBARS was calculated using the absorption coefficient of 155 mM^{-1} cm^{-1}, and the results were expressed as nmol MDA-TBA g FW^{-1}.

Determination of hydrogen peroxide content and ascorbate redox states

Hydrogen peroxide content was measured by the titanium tetrachloride method (Brennan and Frenkel, 1977). Fresh leaf samples were macerated with liquid N_2 in presence of 5% (w/v) TCA and centrifuged at 12,000 *g* (4 °C), and the supernatant was immediately used for H_2O_2 determination. The measurement was performed after reaction of the titanium reagent with H_2O_2 and formation of the hydroperoxide-titanium complex. The H_2O_2 content was calculated from a standard curve and read at 415 nm. The results were expressed as μmol H_2O_2 g^{-1} FW. The contents of reduced (AsA) and total (AsA + DHA) ascorbate were assayed according to Kampfenkel et al. (1995). The assay of reduced ascorbate is based on the reduction of Fe^{3+} to Fe^{2+} by AsA and total ascorbate was measured after complete reduction with 10 mM DTT and 0.5% (w/v) N-ethylmaleimide. The Fe^{2+} complex with 2,2`-bipirydyl was read at 525 nm in a spectrophotometer.

Preparation of crude extract and cytosol and chloroplast enriched fractions

Crude extracts were prepared by grinding fresh leaf samples to a fine powder in liquid N_2 in a mortar and pestle and extracting the powder with ice-cold 100 mM K-phosphate buffer, pH 6.8, containing 0.1 mM EDTA and 2 mM ascorbic acid. Cytosol and chloroplast enriched fractions were obtained according to Cuello and Quiles (2007) with minor modifications. All of the steps were carried out at low temperature (4 °C). Fresh leaf samples (2 g) were homogenized in the absence of liquid N_2 in a medium containing 20 mL of an ice cold extraction buffer (25 mM HEPES; pH 7.6) containing 0.35 M sucrose, 2 mM Na_2-EDTA, 2 mM ascorbic acid, 4 mM DTT and 10 mM $MgCl_2$. After filtration through cheesecloth, the homogenate was centrifuged at 200 *g* for 5 min. The first pellet was discarded, and the supernatant was centrifuged at 2,500 *g*. The pellet containing chloroplasts was then isolated, and the supernatant was centrifuged at 20,000 *g* for 30 min to obtain the cytosol enriched fraction (Asada, 1992). This fraction also contains vacuolar and apoplast fluids.

To diminish cytosolic contamination, the chloroplastic pellet was washed twice with the same extraction buffer. The chloroplastic proteins were extracted by maceration of the pellet with 100 mM K-phosphate buffer at pH 7.0 containing 2 mM ascorbic acid, 4 mM DTT and 10 mM $MgCl_2$. After centrifugation at 15,000 *g* for 15 min, the chloroplastic fraction was obtained. The protein content in each fraction was measured by the Bradford (1976) method using BSA as standard.

Enzyme activity assays

The ascorbate peroxidase (APX; EC 1.11.1.1) and gluthatione peroxidase (GPX; EC 1.11.1.9) activities were measured in cytosolic and chloroplast fractions. The catalase (CAT; EC 1.11.1.6) and superoxide dismutase (SOD; EC 1.15.1.1) activities were determined in the crude extract. The APX activity was measured by following the ascorbate oxidation by the decrease in absorbance at 290 nm (Nakano and Asada, 1981) with minor modifications. The cytosolic and chloroplast APX activities were assayed in a reaction mixture containing 0.5 mM ascorbate and 0.1 mM EDTA dissolved in 100 mM K-phosphate buffer, pH 7.0, and enzyme extract. The reaction was started by adding 30 mM H_2O_2. The enzyme activity was measured by the decrease in absorbance at 290 nm, 25 °C, for 300 s. To avoid interference by type III peroxidase activity, two parallel determinations were performed: in the absence (A) and in the presence of pCMB (B), a specific APX inhibitor (Amako et al., 1994). The net APX activity was calculated from the difference, A-B, and it was expressed as μmol H_2O_2 mg protein^{-1} min^{-1}.

The GPX activity in the cytosolic and chloroplast fractions was measured according to the method of Awasthi et al. (1975) using cumene hydroperoxide as a substrate. Samples (0.1 mL) of the supernatant were mixed with a reaction mixture consisting of 4 mm GSH, 0.2 mm NADPH, 0.05 U of GR (type II from wheat; Sigma, St Louis, MO, USA) and 0.5 mm substrate in phosphate buffer (0.1 M; pH 7.0). The GPX activity was determined by the decrease of NADPH absorption at 340 nm. The non-specific NADPH decrease was corrected for using additional measurements without substrate. The GPX activity was estimated from the molar

extinction coefficient of NADPH (6.22 mm^{-1} cm^{-1}) and expressed as mmol NADPH mg $protein^{-1}$ min^{-1}.

The CAT activity was measured by following the oxidation of H_2O_2 at 240 nm. The CAT activity was determined from the reaction of the crude extract in the presence of 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM H_2O_2. The reaction occurred at 30 °C, and the absorbance was monitored at 240 nm for 300 s (Havir and McHale, 1987). The CAT activity was calculated according to the molar extinction coefficient of H_2O_2 (36 mM^{-1} cm^{-1}) and expressed as µmol H_2O_2 mg $protein^{-1}$ min^{-1}.

The SOD activity was determined by the inhibition of blue formazan production by NBT photoreduction. The SOD activity was measured by adding leaf extract to a mixture containing 50 mM K-phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM L14 methionine, 2 µM riboflavin, and 75 µM *p*-nitro blue tetrazolium chloride (NBT) in the dark. The reaction was carried out under illumination (30 watt fluorescent lamp) at 25 °C for 6 min. The absorbance was measured at 540 nm (Giannopolotis and Ries, 1977). One SOD activity unit (U) was defined as the amount of enzyme required to inhibit 50% of the NBT photoreduction, and the activity was expressed as U mg $protein^{-1}$ min^{-1} (Beauchamp and Fridovich, 1971).

Protein extraction

Leaves of 4-month-old non-transformed and knockdown plants were collected and macerated in liquid N_2, homogenized in buffer (0.5 M Tris-HCl, pH 8.3, 2% Triton X-100, 20 mM $MgCl_2$, 2% β-mercaptoethanol, 1 mM PMSF, 2.5% PEG and 1 mM EDTA) and incubated at 4°C for 2 hours. The supernatant was added to a solution containing 10% trichloroacetic acid (TCA) in acetone (w/v), and the mixture was incubated for 12 hours at -20 °C to precipitate the protein. The resulting precipitate was washed in cold acetone and dissolved in a solution containing 7 M urea and 2 M thiourea (Acquadro et al., 2009). The extracted proteins were quantified by the Bradford method (Bradford, 1976).

2D Gel and image analysis

Approximately 500 mg of protein extract was used for two-dimensional electrophoresis analyses. In the first dimension, proteins were separated according to their isoelectric points using a gradient of pH 4 to 7. In the second dimension, proteins were separated according to their molecular weight in 12.5% SDS-polyacrylamide gel. The gels were stained with Comassie Blue and analyzed to choose the spots of interest, which were excised from gels and digested with trypsin (Hellman et al., 1995). The solutions containing the peptides were separated by a multidimensional chromatographic system.

The eluted fractions were analyzed in a mass spectrometer characterized by a source of electrospray ionization (ESI), two mass analyzers - a quadrupole (Q) associated with a tube in which one measures the flight time of ions (TOF) and an ion detector. Coupled with the online Q-TOF, a capillary chromatography system (Acquity UPLC) is used, in which the sample will undergo a reverse phase column. To perform MS analysis, the peptides eluted from the column are ionized, and then the ratio is determined on mass charge (m/z) of each. The MS/MS spectra generated were processed by Proteinlynx (Micromass) and compared to the National Center for Biotechnology Information (NCBI) database using the MASCOT software (http//: www.matrixscience.com). Each experiment was repeated three times.

Statistical analysis and experimental design

The experiment was arranged in a completely randomized design in a factorial 2x3 design with two main treatments (transgenic and NT plants), 3 sub-treatments (control, HL and MV) with four independent replicates, each consisting of an individual pot containing three plants. The RT-qPCR and biochemical measurements were performed four times. The data were analyzed using ANOVA, and the mean values were compared using the Tukey's test at a confidence level of 0.05.

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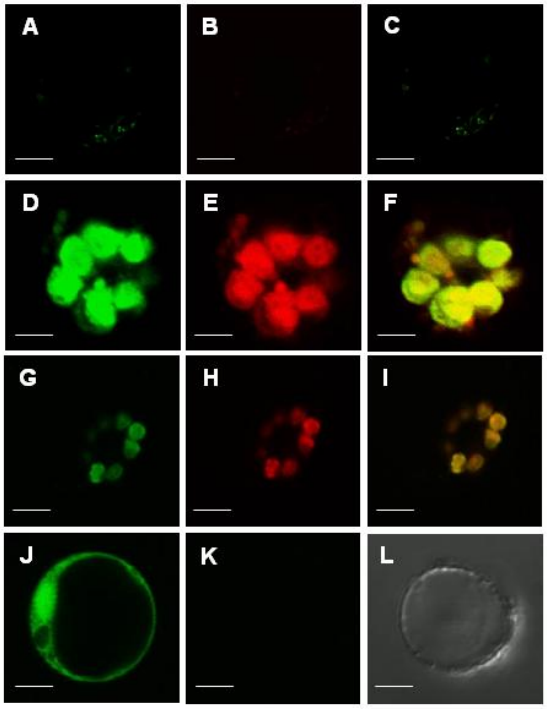

Figure 1. Ascorbate peroxidase proteins (OsAPX5 and OsAPX7) are localized in mitochondria and chloroplasts. Green signals indicate YFP fluorescence, red signals indicate chlorophyll autofluorescence, and yellow signals show the merged images. Subcellular localization of the OsAPX5 protein in mitochondria and chloroplasts in rice protoplasts visualized by transient expression of the 35S-OsAPX5::YFP cassette (A-C) and (D-F), respectively, and in the chloroplasts by 35S-OsAPX7::YFP (G-I). Positive controls, using the 35S::GFP cassette (J), and negative controls (K and L). All scale bars represent 10 µm.

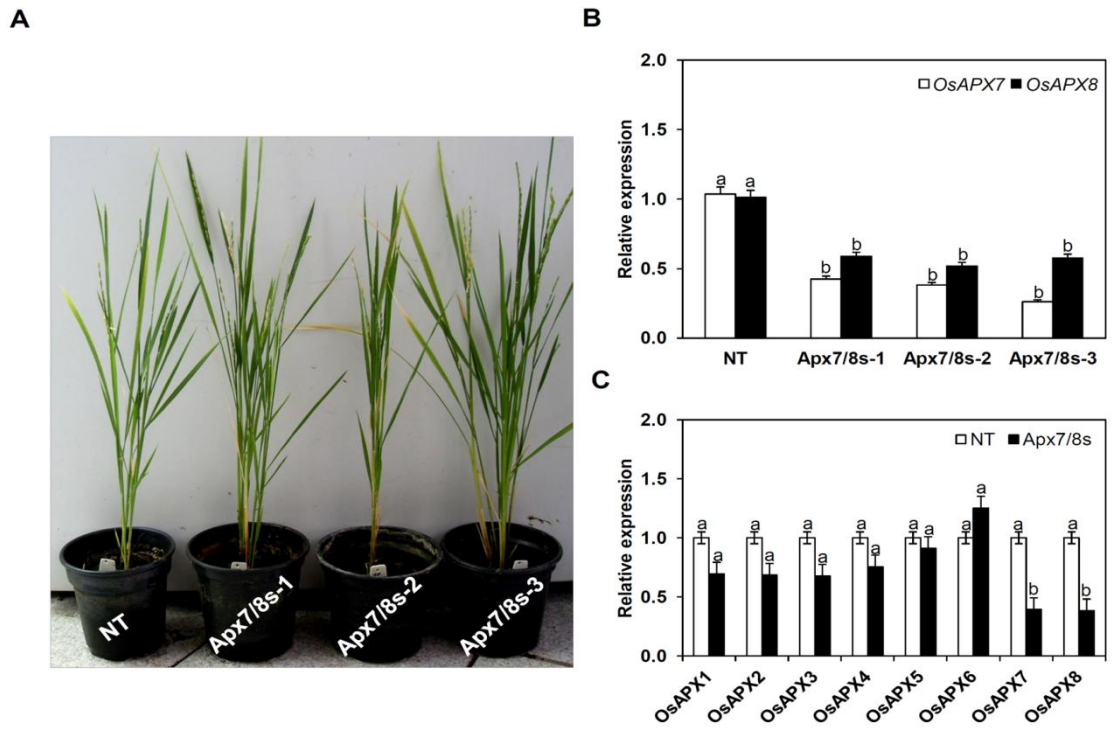

Figure 2. **Characterization of chloroplastic *OsAPX7* and *OsAPX8* genes in Apx7/8s knockdown plants.** (A) Non-transformed (NT) and three lines of Apx7/8s plants grown under controlled conditions; (B) Quantitative determination of *OsAPX7* and *OsAPX8* mRNA in leaves of NT and Apx7/8s plants grown under control conditions. The transcript level of *OsAPX7* and *OsAPX8* present in NT plants was used to normalize the transcript accumulation in Apx7/8s plants and NT; (C) Expression of all *OsAPX* genes in Apx7/8s plants at generation F1 compared to the expression observed in NT plants. The values represent the mean ± SD (N = 3). Each plant line that promotes a significantly different expression of individual APX loci was assigned a specific letter (a or b).

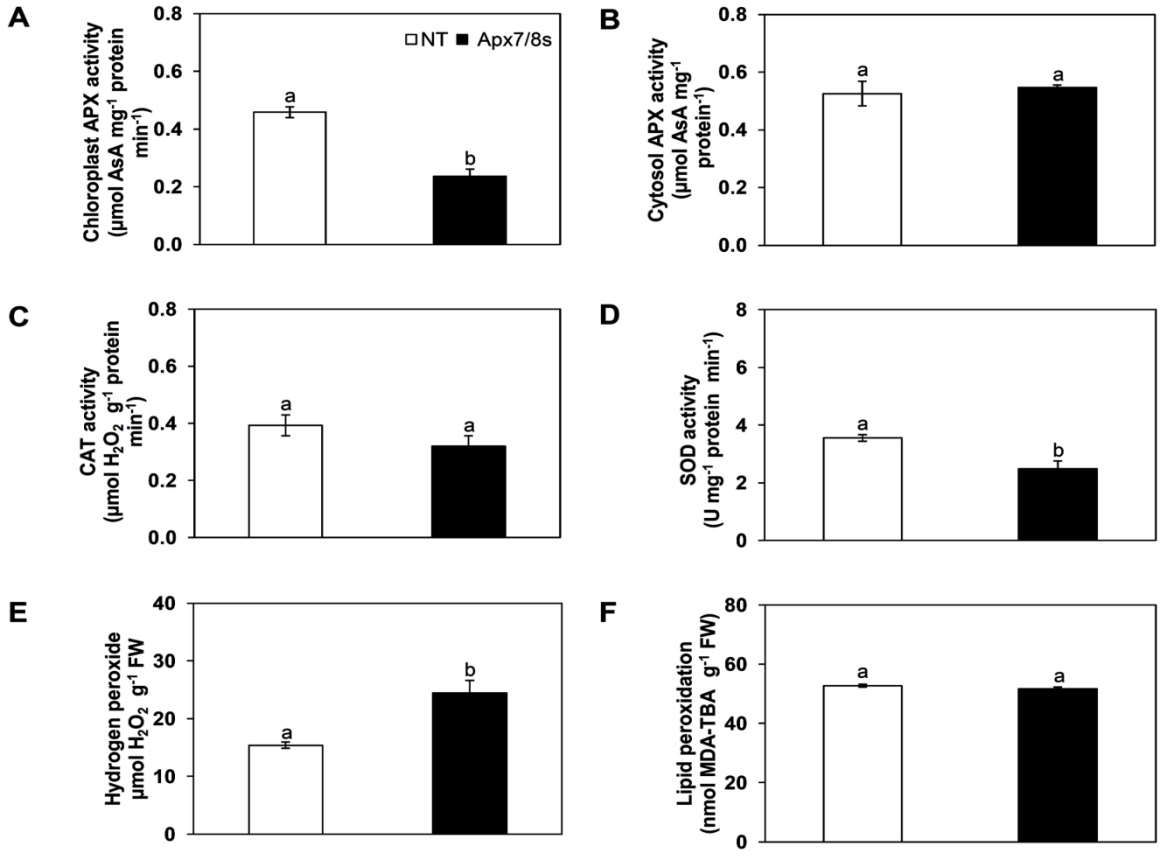

Figure 3. Effect the double silencing of the chloroplastic *OsAPX* genes on the antioxidants enzymes activity, content H_2O_2 and level lipid peroxidation. The ascorbate peroxidase-APX, Chloroplast APX activity (A), Cytosol APX activity (B), catalase-CAT activity (C), superoxide dismutase-SOD activity (D) Content Hydrogen peroxide (E) and level of Lipid peroxidation (F). Determinations were performed in leaves of plants transgenic rice Apx7/8s and non-transformed (NT) plants grown under control conditions. Values represent the mean ± SD (N = 3). Different letters (a and b) at the top of the error bars indicate statistically different means ($P < 0.05$). FW, fresh weight; MDA, malondialdehyde.

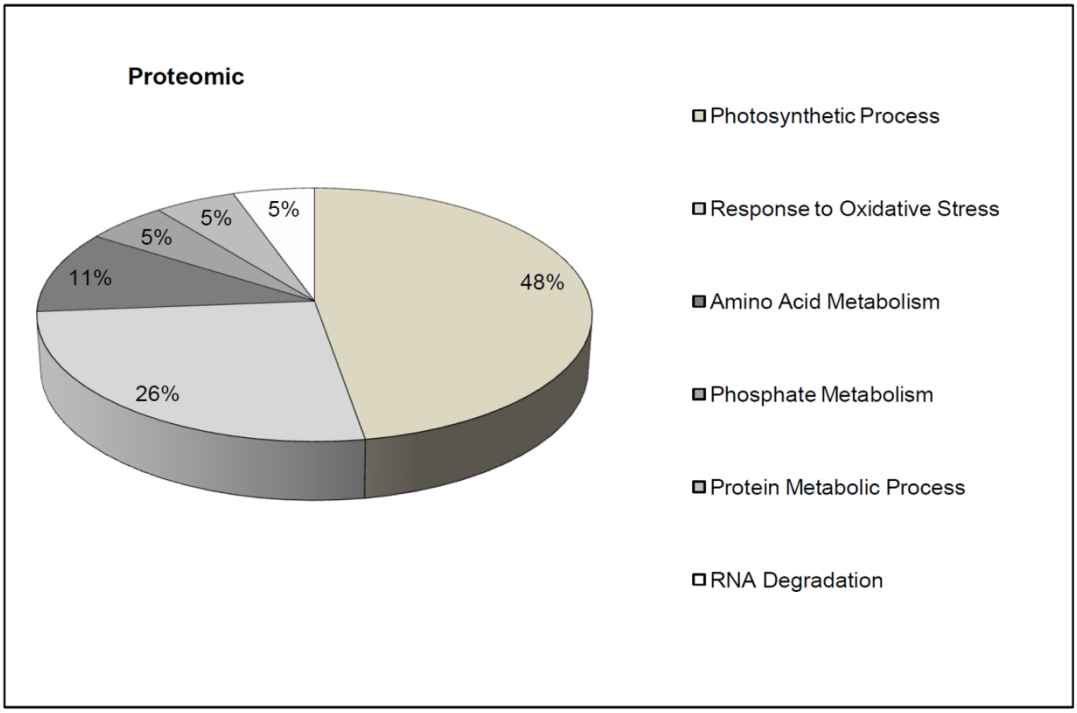

Figure 4. Groups of the differentially expressed proteins detected by mass spectrometry analysis of Apx7/8s knockdown plants. The proteomic analyses were performed in leaves of the Apx7/8s knockdown and non-transformed (NT) plants under control conditions. The values represent the mean ± SD (N = 3).

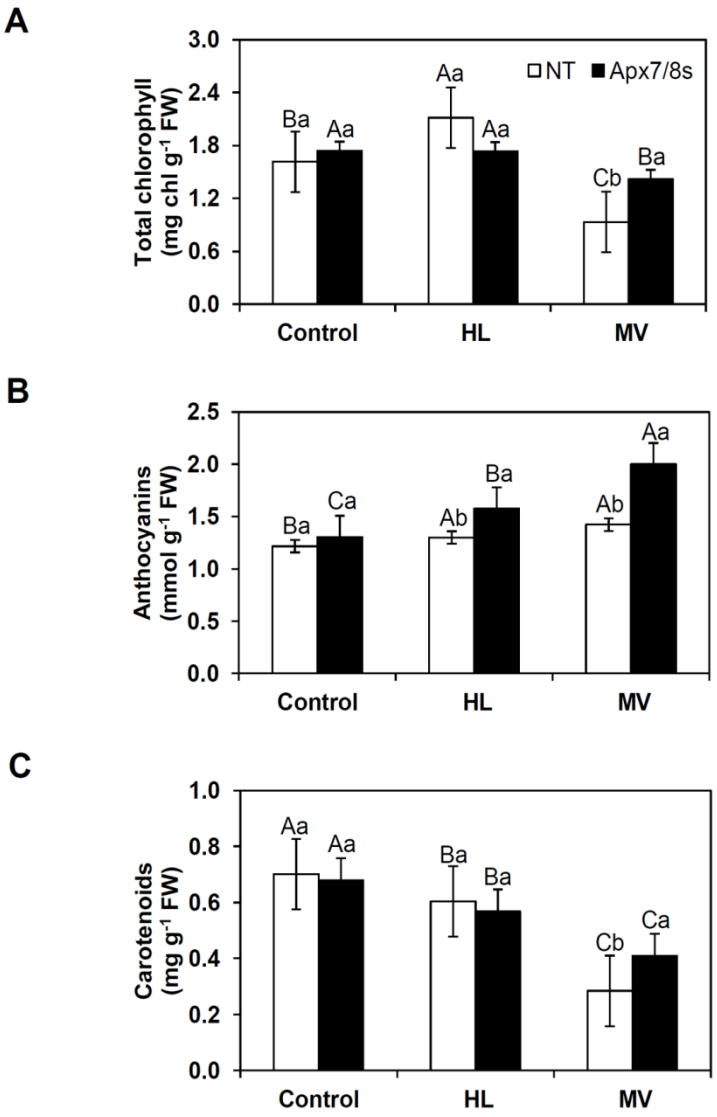

Figure 5. Determination of the total chlorophyll, anthocyanin and carotenoids in plants exposed to abiotic stress. (A) Total chlorophyll, (B) anthocyanin and (C) carotenoids in leaves of Apx7/8s knockdown and non-transformed (NT) plants exposed to control, high light (HL) and methyl viologen (MV) treatments. Different lowercase letters represent significant differences among the Apx7/8s knockdown and NT plants whereas different capital letters represent significant differences among the stress treatments, at a confidence level of 0.05. The data are the means of four replicates and were compared by Tukey's test. FW, fresh weight.

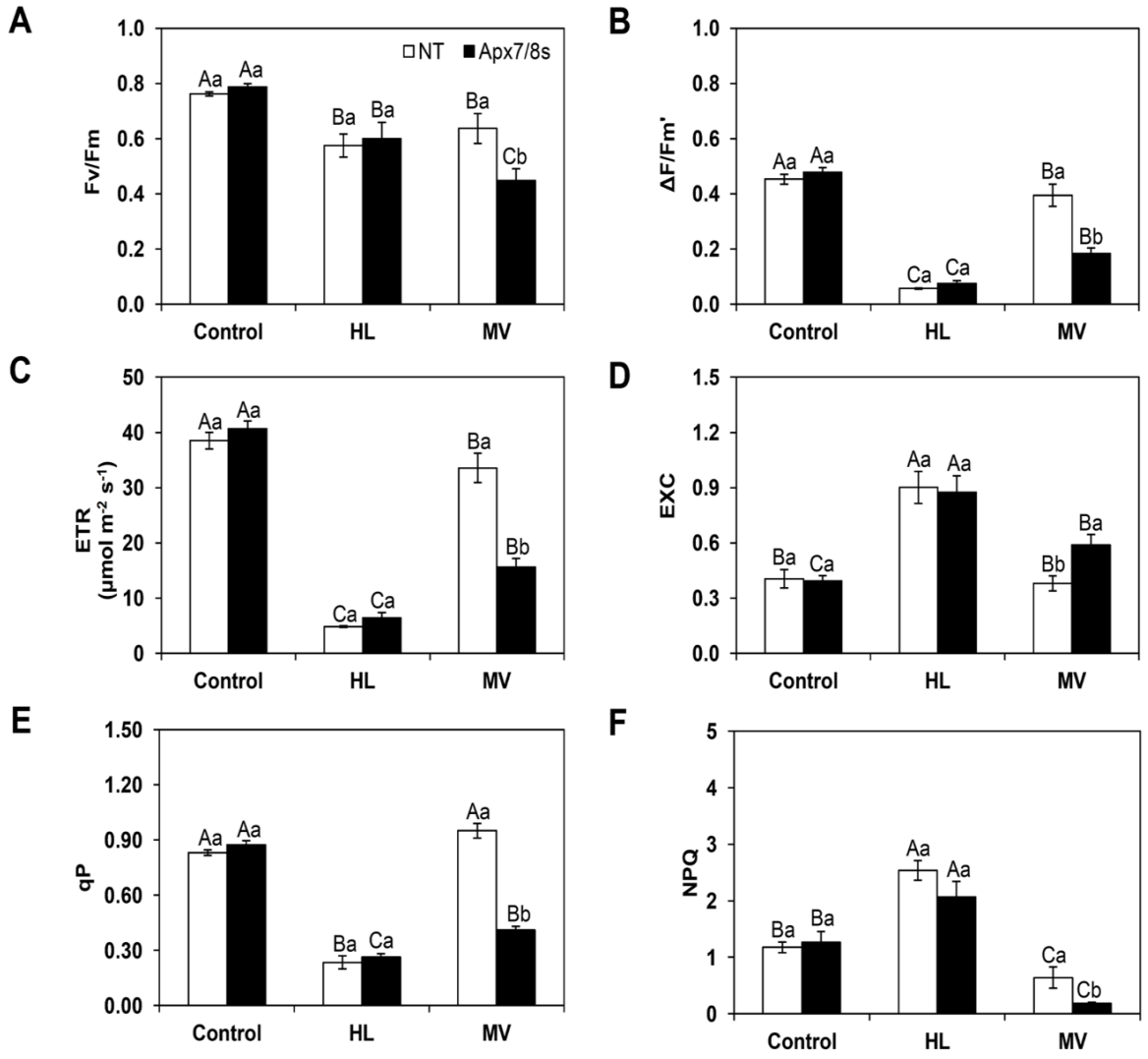

Figure 6. Chlorophyll *a* fluorescence parameters in plants exposed to abiotic stress. (A) Potential (*F*v/*F*m) and (B) effective (Δ*F*/*F*m′) quantum efficiency of photosystem II (C) electron transport rate (ETR), (D) electron excess in the photosystem II (EXC), (E) photochemical quenching (qP) and (F) nonphotochemical quenching (NPQ) in leaves of Apx7/8s knockdown and non-transformed (NT) plants exposed to control, high light (HL) and methyl viologen (MV) treatments. Different lowercase letters represent significant differences among the Apx7/8s knockdown and NT plants whereas different capital letters represent significant differences among the stress treatments, at a confidence of level of 0.05. The data are the means of four replicates and were compared by Tukey's test.

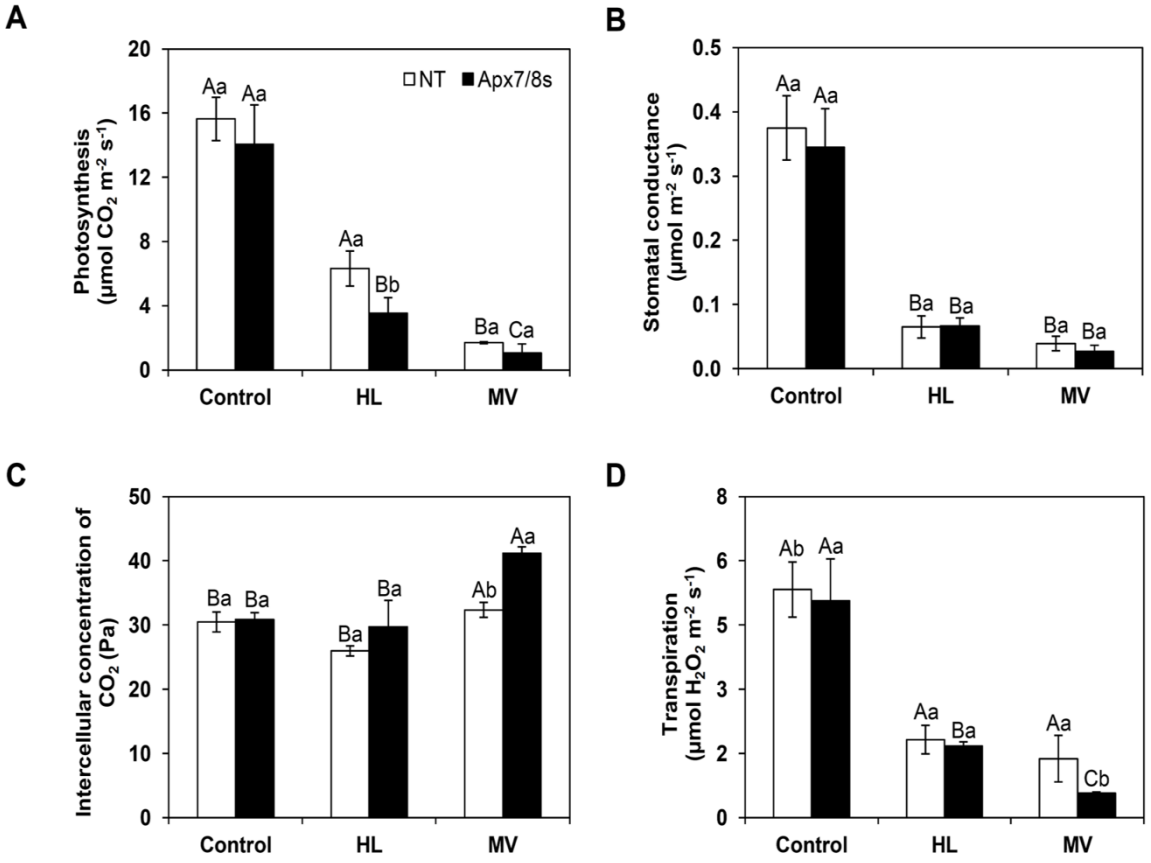

Figure 7. Photosynthetic parameters in plants exposed to abiotic stress. (A) Photosynthetic CO_2-assimilation, (B) stomatal condutance, (C) intercellular concentration CO_2 and (D) transpiration in leaves of Apx7/8s knockdown and non-transformed (NT) plants exposed to control, high light (HL) and methyl viologen (MV) treatments. Different lowercase letters represent significant differences among the Apx7/8s knockdown and NT plants, whereas different capital letters represent significant differences among the stress treatments, at a confidence level of 0.05. The data are the means of four replicates and were compared by Tukey's test.

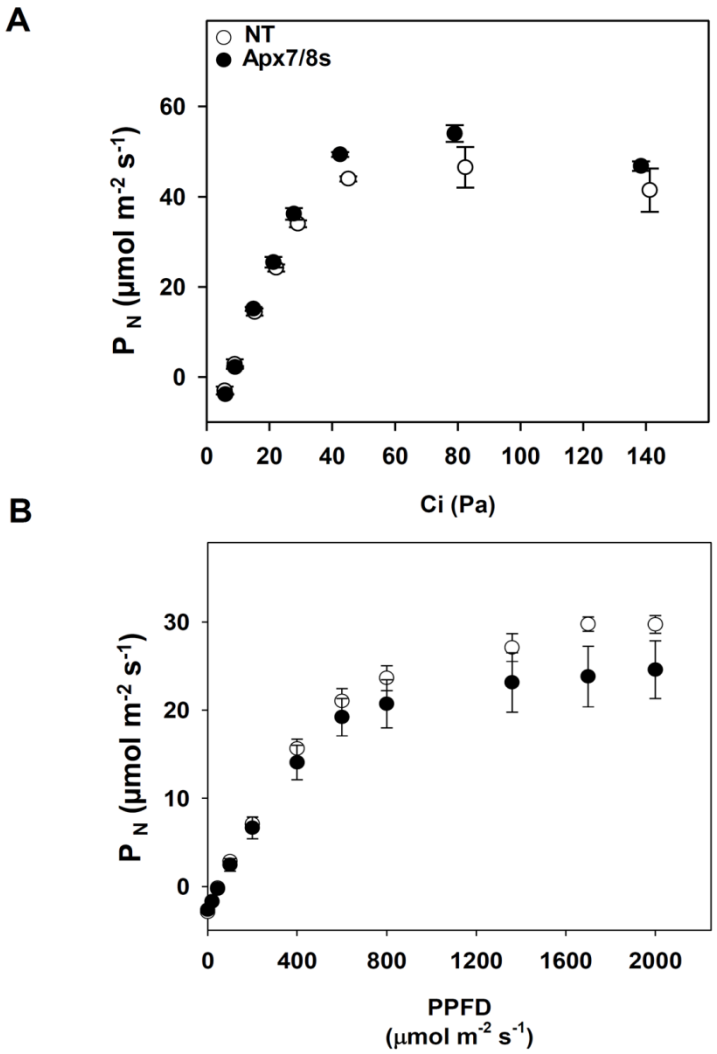

Figure 8. The P_N – Ci and P_N – PPFD fitting curves of the Apx7/8s knockdown and non-transformed (NT) plants. Both types of plants were compared in terms of photosynthetic efficiency under optimum conditions of temperature and light. (A) The P_N – Ci fitting curve (photosynthesis depending of the intercellular concentration of CO_2) and (B) P_N – PPFD fitting curve (photosynthesis depending of light intensity) were performed to calculate some photosynthetic parameters associated with photosynthesis efficiency and calculated by the mathematical model developed by Sharkey et al., (2007) and Lieth and Reynolds (1987).

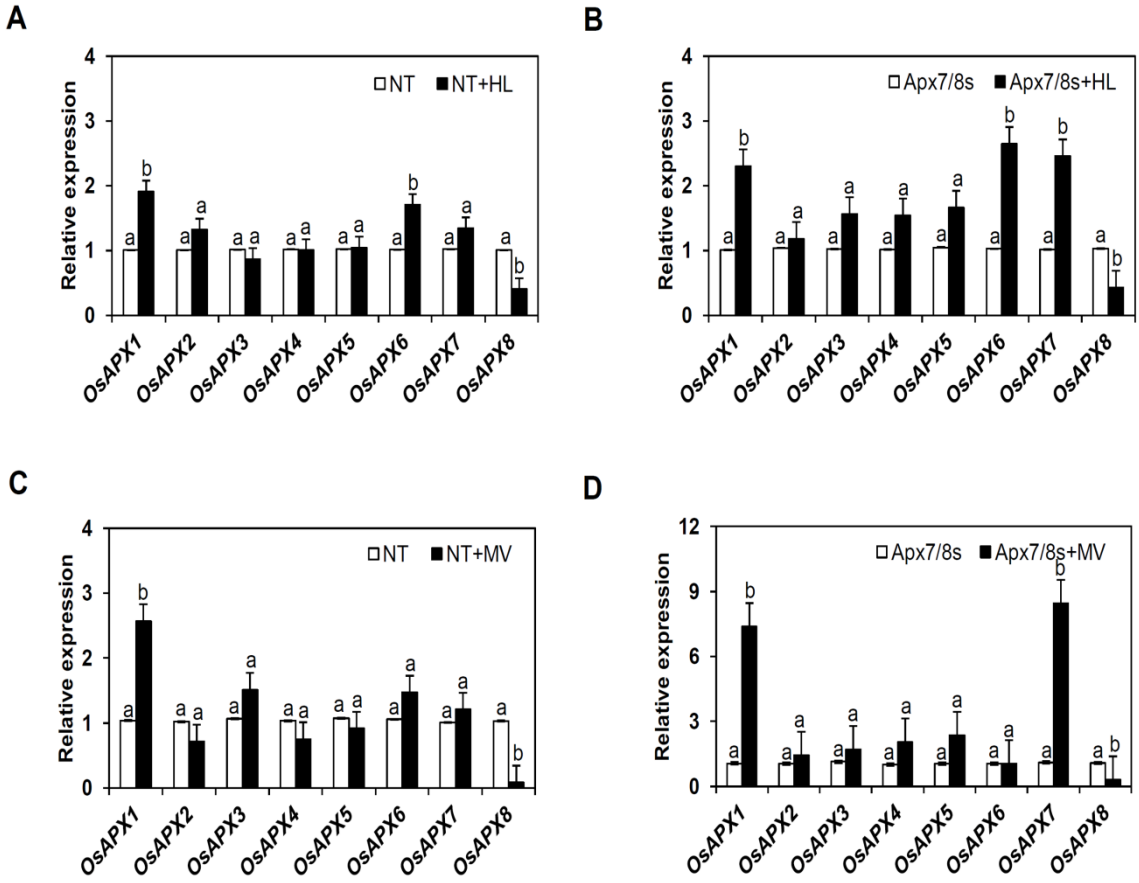

Figure 9. Transcript levels of *OsAPX* genes in plants exposed to control, high light (HL) and methyl viologen (MV) treatment. (A) Non-transformed (NT) plants exposed to HL treatment; (B) Apx7/8s knockdown plants exposed to HL treatment; (C) NT plants exposed to MV treatment and (D) Apx7/8s knockdown plants exposed to MV treatment. Each bar represents the mean of three replicates of four biological samples, with the standard deviation indicated. The relative expression of each APX locus was normalized to the average value obtained from the control plants. Different letters represent significant differences among the Apx7/8s knockdown and NT plants, at a confidence level of 0.05.

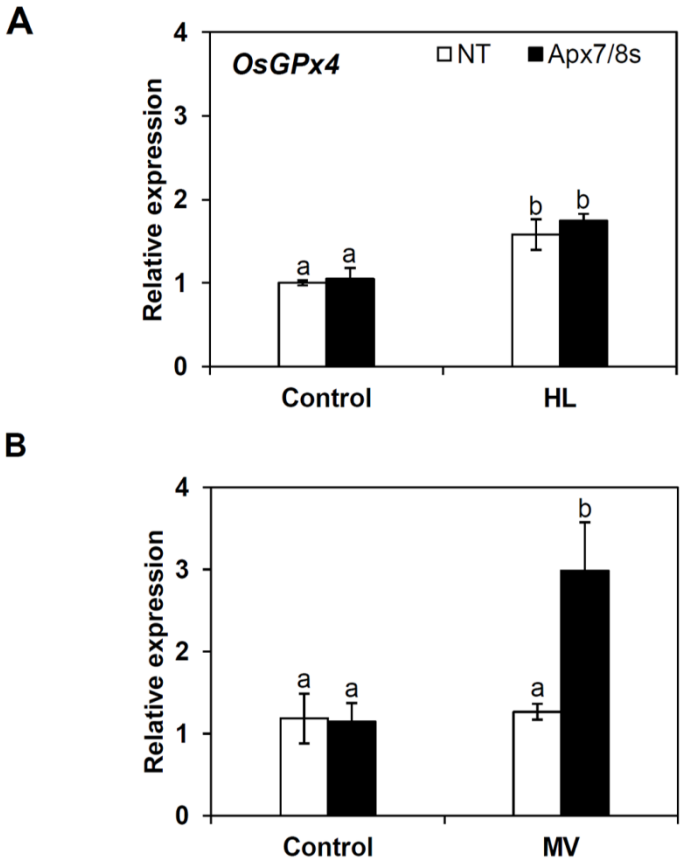

Figure 10. Transcript levels of the *OsGPx4* gene in plants exposed to control, high light (HL) and methyl viologen (MV) treatment. (A) Non-transformed (NT) e Apx7/8s knockdown plants exposed to HL treatment; (B) NT and Apx7/8s knockdown plants exposed to MV treatment. Each bar represents the mean of three replicates of four biological samples, with the standard deviation indicated. The relative expression of *OsGPx4* gene was normalized to the average value obtained from the control plants. Different letters represent significant differences among the Apx7/8s knockdown and NT plants, at a confidence level of 0.05.

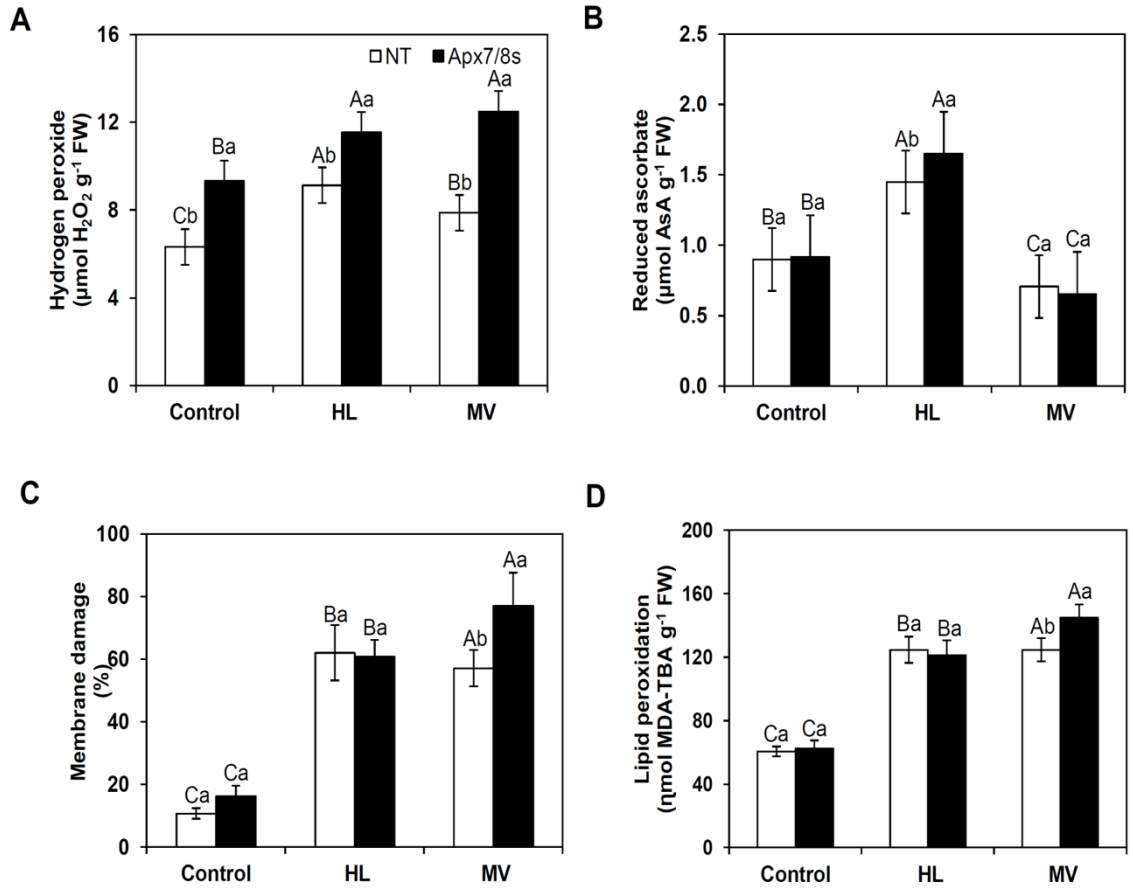

Figure 11. Effect of the double silencing of the chloroplastic *OsAPX* genes on the H_2O_2 content, reduced ascorbate (AsA) content, membrane damage and level lipid peroxidation in plants exposed to abiotic stress. (A) Hydrogen peroxide content, (B) reduced ascorbate (AsA) content, (C) membrane damage (K+ leakage,%) and (C) lipid peroxidation [level of thiobarbituric acid reactive substances (TBARS)] in leaves of Apx7/8s knockdown and non-transformed (NT) plants exposed to control, high light (HL) and methyl viologen (MV) treatments. Different lowercase letters represent significant differences among the Apx7/8s knockdown and NT plants, whereas different capital letters represent significant differences among the stress treatments, at a confidence level of 0.05. The data are the means of four replicates and were compared by Tukey's test. FW, fresh weight; MDA, malondialdehyde.

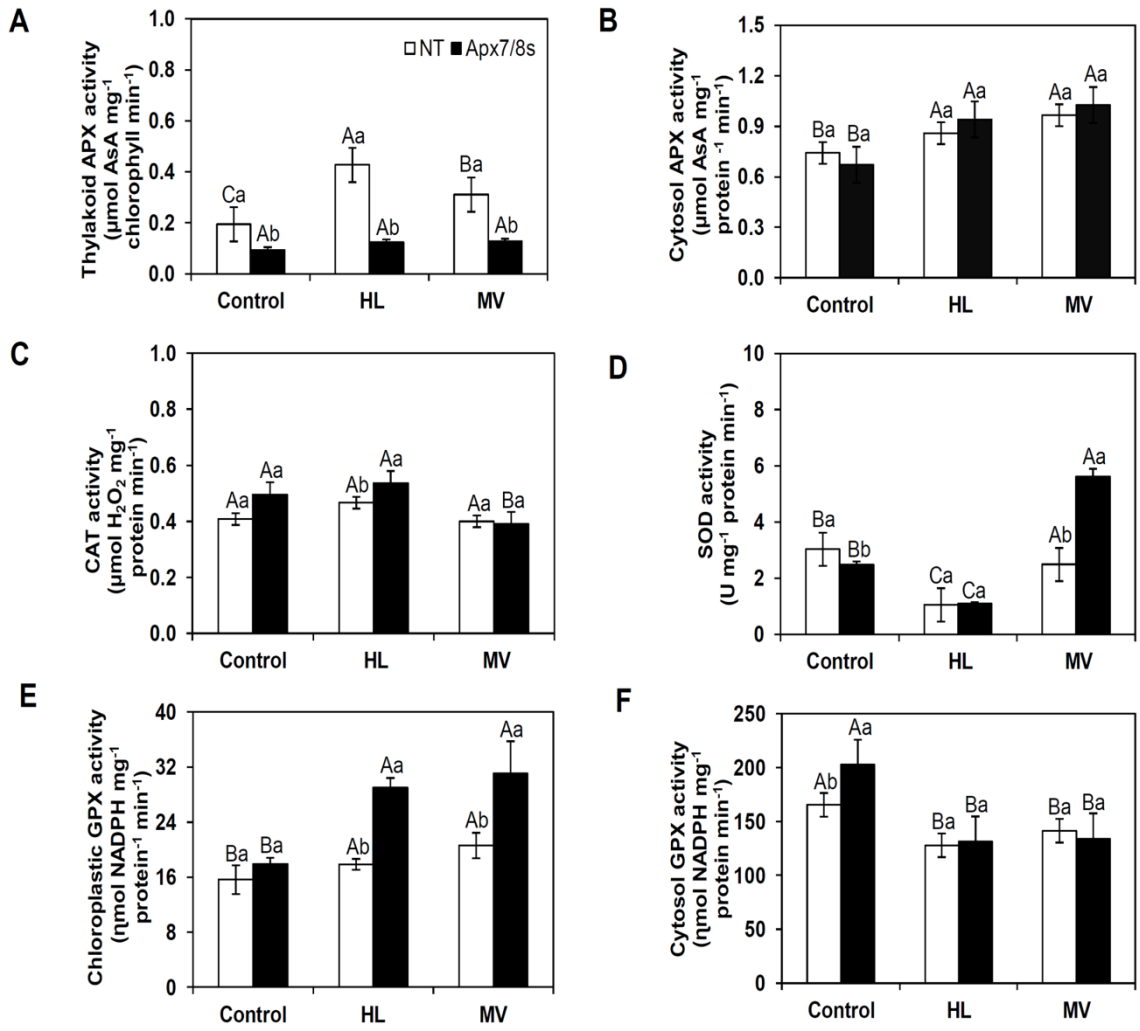

Figure 12. Effect of the double silencing of the chloroplastic *OsAPX* genes on antioxidant enzymatic activity in plants exposed to abiotic stress. Enzymatic activity of (A) thylakoid ascorbate peroxidase (APX); (B) cytosol ascorbate peroxidase (APX); (C) catalase (CAT); (D) superoxide dismutase (SOD); (E) chloroplastic gluthatione peroxidase (GPX) and (F) cytosol gluthatione peroxidase (GPX) in leaves of Apx7/8s knockdown and non-transformed (NT) plants exposed to control, high light (HL) and methyl viologen (MV) treatments. Different lowercase letters represent significant differences among the Apx7/8s knockdown and NT plants, whereas different capital letters represent significant differences among the stress treatments, at a confidence level of 0.05. The data are the means of four replicates and were compared by Tukey's test.

Table 1. Differentially regulated proteins in Apx7/8s knockdown and non-transformed plants identified by mass spectrometer[1].

Functional Classification	Protein Name	Down/Up Regulated	Acession Number (GeneBank)
Photosynthetic Process	Putative, ATP synthase subunit alpha	down	ABF98292
	Oxygen-evolving enhancer protein	down	BAB64069
	Putative ATP synthase like protein	down	AAS90662
	Phosphoribulokinase/Uridine kinase family protein	up	AAM94337
	Rubisco activase small isoform precursor	up	BAA97584
	Glyceraldehyde-3-phosphate dehydrogenase B, chloroplast, putative, expressed	up	NP_001048847
	Phosphoglycerate kinase	up	BAH91316
	Putative fructose-biphospate aldolase isozyme	up	AXX95072
	Putative, Ferredoxin-NADP(H) oxidoreductase	up	BAG99040
Response to Oxidative Stress	Heat shock protein	down	NP_001052417
	Expressed protein	down	NP_001064363
	Putative superoxide dismutase [Cu-Zn], chloroplast precursor	down	BAD09607
	L- ascorbate peroxidase 1, cytosolic	up	NP_001049769
	L- ascorbate peroxidase 2, cytosolic	up	BAA08264
Amino Acid Metabolism	Putative, Glycine dehydrogenase	up	BAD35509
	Putative, glycine cleavage system H protein	up	AAK39504
Phosphate Metabolism	Putative inorganic pyrophosphatase	up	BAD16934
Protein Metabolic Process	Putative, AAA-type ATPase family protein	up	AAS90662
RNA Degradation	Drought-induced S-like ribonuclease	up	AAL33776

[1]Functional classification, protein name, down or up-regulated protein and accession number (GeneBank) in Apx7/8s knockdown plants are shown.

Table 2. Parameters associated with photosynthetic efficiency in Apx7/8s knockdown and non-transformed (NT) plants obtained from P_N-Ci and P_N-PPFD fitting curves models[1]. The data represent the means of three replicates and different letters indicate differences among plant types at a confidence level of 0.05 by Tukey´s test.

Photosynthetic parameters	NT	Apx7/8s
Vcmax	208±11.32 b	244±13.64a
Jmax	253±14.56b	299±17.95a
TPU	16±1.2a	18±1.5a
P_N max (CO_2)	46±2.7b	55±3.34a
Light respiration (Rd)	5.6±0.78b	7.7±0.98a
Photorespiration (Pr)	7.9±0.5b	10.3±0.9a
CO_2-compensation point (I^*)	3.8±0.36b	5.0±0.52a
Quantum effciency (α)	0.0577±0.004a	0.0504±0.002b
P_N max (light)	33±3.22a	26±1.31b
Dark respiration (Rn)	2.86±0.32a	2.62±0.36a
Convexity (θ)	0.75±0.08a	0.86±0.09a
Light-compensation point (I^*)	48±6.34a	49±5.78a

[1]Photosynthetic parameters: Vcmax – Rubisco maximum carboxylation rate; Jmax – maximum electron transport rate; TPU - triose phosphate use; P_N max (CO_2) maximum rate of CO_2 assimilation under CO_2 saturation conditions; P_N max (light) maximum rate of CO_2 assimilation under light saturation.

3. DISCUSSÃO GERAL

Espécies reativas de oxigênio são constantemente produzidas no metabolismo aeróbico e são geradas principalmente em cloroplastos e mitocôndrias devido à alta atividade metabólica dessas organelas. O trabalho coordenado dos mecanismos de controle de ERO é essencial para a proteção da planta de danos oxidativos que podem levar à morte celular (Mittler, 2002; Mittler et al., 2004; Scandalios, 2005). A enzima APX é fundamental para a defesa das plantas contra o estresse oxidativo, atuando na eliminação de ERO juntamente com os demais componentes do sistema antioxidante.

Com o intuito de discutir os principais achados em relação ao papel de proteção das enzimas ascorbato peroxidases contra estresses ambientais em diferentes espécies de plantas, um levantamento das principais publicações relacionadas à regulação da APX foi realizado. Na pesquisa realizada na base de dados “ISI Web of Knowledge” os resultados claramente demonstraram que o número de publicações relacionadas à enzima APX fortemente aumentou nos últimos dez anos, bem como publicações relacionadas às demais enzimas envolvidas no sistema antioxidante e as espécies reativas de oxigênio em sistemas vegetais (Figura 1, capítulo 1). Esses dados mostram a importância do metabolismo antioxidante vegetal, e que cada vez mais pesquisas vêm sendo desenvolvidas visando compreender as funções exercidas por essas enzimas em diferentes espécies de plantas. Entretanto, as pesquisas referentes à regulação das isoformas citosólicas e cloroplastídicas de APX são amplamente mais abordadas na literatura, enquanto que, os estudos envolvendo as isoformas peroxissomais de APX ainda são poucos explorados.

Vários fatores de estresses impostos nos tecidos vegetais induzem mudanças no metabolismo do oxigênio que causa estresse oxidativo. A regulação das APXs em resposta a estresses como temperaturas extremas, alta luminosidade, seca, salinidade e metais pesados foi abordada. Nos diferentes estresses foram observadas distintas respostas para as APXs, tanto ao nível de transcritos quanto de proteína e/ou atividade enzimática. Diferentes respostas também foram observadas para outras enzimas importantes do metabolismo

antioxidante, como SOD e CAT. No entanto, o conjunto de dados publicados até o momento envolvendo distintas abordagens, tais como plantas mutantes deficientes em um gene ou mais de *APX*, ou superexpressando diferentes genes de *APX* ou até mesmo superexpressando dois ou três genes de enzimas antioxidantes de uma única vez, como *SOD, APX* e *DHA* demonstram que o sistema antioxidante é muito complexo e que as enzimas APX juntamente com os demais componentes do sistema antioxidante, sejam eles enzimáticos ou não enzimáticos, exercem funções fundamentais na proteção de plantas contra o estresse oxidativo.

O conhecimento da localização subcelular de uma proteína auxilia na compreensão de funções celulares como um todo (Heazlewood et al., 2005). Desse modo, a localização subcelular de sete proteínas de OsAPX de arroz foi investigada experimentalmente *in vivo* visando determinar o compartimento celular no qual cada isoforma está localizada. Assim fusões traducionais do cDNA de *OsAPX1*, *OsAPX2*, *OsAPX3*, *OsAPX4*, *OsAPX5*, *OsAPX6* e *OsAPX7* com a proteína marcadora YFP foram construídas pelo sistema Gateway^{TM} Technology (Invitrogen) e utilizadas na transformação de protoplastos isolados de folhas jovens de plantas de arroz. O sistema de expressão transiente em protoplastos de arroz foi escolhido pela rapidez na obtenção de células transformadas e a simples visualização por microscopia de confocal. Nossos resultados corroboram aqueles previamente obtidos pelo nosso grupo com programas de predição de localização subcelular *in silico* e experimentalmente utilizando células BY-2 de tabaco (Teixeira et al, 2004; 2006; Teixeira, 2005), onde as proteínas OsAPX1 e OsAPX2 são localizadas no citosol, OsAPX3 e OsAPX4 nos peroxissomos (Figura 2, capítulo 2), OsAPX5 no cloroplasto, OsAPX6 na mitocôndria e OsAPX7 no cloroplasto (Figura 1, capítulo 3).

No entanto, existem proteínas que são codificadas por um único gene e são transportadas para mais de uma organela (Mackenzie, 2005), assim nossos resultados em protoplastos de arroz também revelaram que o gene *OsAPX5* codifica uma proteína de duplo direcionamento. APX5 de arroz foi encontrada no cloroplasto, conforme já havia sido predito, e na mitocôndria (Figura 1, capítulo 3). Além disso, nossos dados em protoplastos de arroz confirmaram as predições

iniciais que OsAPX1 e OsAPX2 são localizadas no citosol e também demonstram a localização dessas proteínas no núcleo celular (Figura 2, capítulo 2). Entretanto, mais experimentos são necessários para comprovar a localização subcelular dessas proteínas também no núcleo celular em arroz.

A estratégia utilizada para estudar a função dos genes cloroplastídicos de *OsAPX* foi o silenciamento por RNA de interferência. Visando obter a redução da expressão de ambos os genes cloroplastídicos simultaneamente buscou-se regiões conservadas entre os genes *OsAPX7* e *OsAPX8.* Os genes *OsAPX7* e *OsAPX8* foram silenciados usando construções repetidas e invertidas transcrevendo o dsRNA (do inglês "*hairpin*"). Para o silenciamento simultâneo dos dois genes uma região transcrita do gene *OsAPX7* de 238 pb foi escolhida (Apêndice 2) a qual possui 81% de similaridade com o gene *OsAPX8* e 34 nucleotídeos contínuos idênticos à sequência do gene *OsAPX8* (Apêndice 3) o que levaria ao silenciamento pós-transcricional. Conforme previamente relatado (Eamens et al., 2008) um trecho de pelo menos 21 nucleotídeos contínuos com uma combinação perfeita entre a construção "hairpin" e o gene alvo é necessário para dirigir o silenciamento do gene. As regiões escolhidas para o silenciamento dos genes-alvo, apresentam no máximo 13 nucleotídeos contínuos idênticos nos outros genes da família de *OsAPX*, o que teoricamente evita o silenciamento pós-transcricional por RNAi destes genes.

Após a confirmação do silenciamento, uma abordagem integrada, envolvendo a expressão dos genes de *OsAPX*, proteômica e análises bioquímicas e um estudo detalhado da fotossíntese das plantas de arroz silenciadas para os dois genes *chlAPXs* foram realizadas. A expressão dos genes *OsAPX7* e *OsAPX8* foi reduzida em cerca de 60%, consequentemente a atividade da APX diminuiu e um forte aumento no nível de H_2O_2 foi observado nas plantas silenciadas, porém essas plantas não apresentaram alterações fenotípicas visíveis (Figura 2, capítulo 3). Entretanto a análise proteômica dessas plantas revelou que várias rotas envolvidas em importantes processos foram afetadas, devido o silenciamento dos genes cloroplástidicos de *OsAPX*. Uma das principais rotas afetadas foi à envolvida com processos fotossintéticos, onde várias proteínas tiveram sua expressão aumentada (Tabela 1, capítulo 3), seguido de

outras envolvidas com estresse oxidativo, metabolismo de aminóacidos, metabolismo do fosfato, proteínas envolvidas com processos metabólicos e degradação de RNA.

As plantas silenciadas (geração T1) foram submetidas a estresses de alta luz e de MV, um potente gerador de ERO. Devido às alterações causadas pelo estresse aos quais essas plantas foram submetidas, ocorreram danos no fotossistema II, tais como, fotodanos, fotoinibição e bloqueio do transporte de elétrons, de maneira mais acentuada nas plantas silenciadas, em comparação às plantas não transformadas. No entanto, em plantas submetidas à alta luz, os genes cloroplastídicos de *OsAPX* parecem não serem essenciais na proteção de plantas de arroz aos fotodanos, fotoinibição e danos oxidativos induzidos por alta luz no fotosistema II. Por outro lado nossos resultados claramente mostraram que as APXs cloroplastídicas são essenciais na proteção fotossintética, quando plantas de arroz foram submetidas ao tratamento com MV (Figura 6 e 7, capítulo 3).

A expressão de alguns genes da família de *OsAPX* e do gene cloroplastídico *OsGPx4* foi fortemente induzida nas plantas silenciadas submetidas à alta luz e MV, assim como a atividade de duas importantes enzimas SOD e GPX (Figura 9, 10 e 12), porém essas alterações não foram suficientes para desencadear um mecanismo compensatório de proteção dessas plantas de arroz contra estresses abióticos como observado para outras peroxidases (Bonifacio et al., 2011). Assim nossos dados revelam a importância das chlAPXs no metabolismo antioxidante de arroz em condições estresses abióticos.

4. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

No presente trabalho apresentamos uma revisão sobre a regulação da expressão dos genes das enzimas APXs em condições normais e submetidas a estresses abióticos, contribuindo com uma visão geral dos principais resultados publicados até o momento sobre esses genes e as diferentes abordagens que vêm sendo utilizadas no estudo das funções de proteção oxidativa desempenhadas pelas APXs.

A confirmação da localização subcelular de sete das oito isoformas de OsAPX permitirá um maior entendimento da função desempenhada de cada APX em arroz. Entretanto, para as isoformas OsAPX1 e OsAPX2 mais experimentos são necessários, para comprovar a localização dessas proteínas também no núcleo celular em arroz. Experimentos de imunolocalização serão realizados, para os quais anticorpos específicos já se encontram disponíveis.

A estratégia adotada para o estudo funcional dos genes *OsAPX7* e *OsAPX8* nos permitiu um maior entendimento da função exercida por esses genes em uma planta monocotiledônea, uma vez que os vários estudos até então apresentados com essas isoformas são concentrados em *Arabidopsis*. As diferentes abordagens utilizadas nos permitiram avaliar a função exercida pelas isoformas chlAPXs em plantas de arroz submetidas a estresses por alta luz e metil viológeno, potentes geradores de ERO. Os resultados claramente mostram a importância dessas enzimas na proteção fotossintética da planta em condições de estresses abióticos.

Várias linhagens de plantas silenciadas individualmente para o gene *OsAPX7* e *OsAPX8* encontram-se atualmente disponíveis. O estudo dessas plantas permitirá um melhor entendimento da função específica desempenhada por cada isoforma de chlAPXs individualmente em arroz. Além disso, linhagens de plantas com uma construção expressando os genes repoteres GFP e GUS foram também produzidas para estudos dos promotores dos genes *OsAPX7* e *OsAPX8*.

Devido às similaridades nas sequências, estrutura, ordem e funções de genes entre os diversos cereais, genes identificados em arroz são importantes

agronomicamente, pois o entendimento da função de genes de arroz pode diretamente ser aplicado a outros cereais (Upadadhaya, 2006). Dessa forma, os resultados aqui apresentados podem contribuir para o entendimento da funcionalidade das enzimas APXs e sua proteção no metabolismo antioxidante de outros cereais.

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APÊNDICE 1

Seqûências de cDNA dos genes de OsAPX utilizadas para as construções de localização subcelular

OsAPX1 – (código de acesso no NCBI: D45423)
Sequência de cDNA completo:

ATGGCTAAGAACTACCCCGTCGTGAGCGCCGAGTACCAGGAGGCCGTCGAGAAGG
CCAGGCAGAAGCTGCGCGCCCTCATCGCCGAGAAGAGCTGCGCCCCTCTCATGCTC
CGCCTCGCGTGGCACTCGGCGGGGACGTTCGACGTGTCGTCGAAGACCGGGGGCC
CGTTCGGGACGATGAAGACCCCGGCGGAGCTGTCGCACGCCGCCAACGCGGGGCT
GGACATCGCGGTGCGGATGCTCGAGCCCATCAAGGAGGAGATACCCACCATCTCCT
ACGCCGATTTCTACCAGCTTGCCGGAGTTGTGGCCGTCGAGGTGTCCGGTGGACCT
GCCGTCCCCTTCCACCCAGGAAGGGAGGACAAACCTGCACCCCCACCTGAGGGCC
GTCTTCCTGATGCTACCAAGGGTTCTGACCACCTAAGGCAGGTCTTCGGTGCGCAGA
TGGGCTTGAGTGATCAGGACATTGTTGCCCTCTCTGGCGGTCACACCCTGGGAAGG
TGCCACAAGGAAAGATCTGGTTTTGAGGGACCTTGGACAAGAAACCCTCTGCAGTTT
GACAACTCTTACTTCACGGAGCTTCTGAGTGGTGACAAGGAGGGCCTTCTTCAGCTT
CCTAGTGACAAAGCCCTGCTGAGTGACCCTGCCTTCCGCCCACTCGTCGAGAAATAT
GCTGCAGATGAGAAGGCTTTCTTTGAAGACTACAAGGAGGCCCACCTCAAGCTCTCC
GAACTGGGGTTCGCTGATGCTTAA

OsAPX2 – (código de acesso no NCBI: AB053297)
Sequência de cDNA completo:

ATGGGCAGCAAGTCGTACCCGACGGTGAGCGATGAGTACCTGGCGGCCGTGGGCA
AGGCGAAGCGCAAGCTCCGCGGCCTCATCGCCGAGAAGAACTGCGCCCCACTCAT
GCTCCGCCTCGCGTGGCACTCTGCTGGCACCTTCGATGTGTCGTCGAGGACCGGCG
GGCCCTTCGGCACCATGAAGAACCCCGGCGAGCAGTCCCACGCCGCCAACGCCGG
CCTCGACATCGCCGTCAGGCTTCTCGACCCCATCAAGGACCAACTTCCCATCCTCTC
CTACGCCGACTTCTACCAGCTTGCTGGCGTTGTGGCCGTCGAGGTCACCGGCGGAC
CTGAGGTCCCCTTCCATCCGGGCAGGCAGGACAAGCCTGAGCCTCCTCCTGAAGGC
CGTCTTCCTGATGCCACACAAGGTTCTGACCACCTAAGGCAGGTCTTTTCTGCGCAG
ATGGGTTTGAGTGACAAGGACATAGTTGCTCTTCTGGTGGTCACACCCTGGGAAGAT
GCCACAAGGAGAGATCTGGCTTTGAGGGAGCCTGGACGTCCAACCCTTTGATCTTTG
ACAACTCTTACTTCACCGAGCTTGTGAGTGGCGAGAAGGAAGGCCTTCTTCAGCTGC
CAAGTGACAAAGCCCTCATGGCTGACCCAGCCTTCCGTCCACTGGTGGAGAAATATG
CTGCGGATGAGGACGCCTTCTTTGCGGATTACGCCGAGGCCCACCTCAAGCTCTCT
GAACTGGGATTTGCTGAGGAATAA

OsAPX3 – (código de acesso no NCBI: AY382617)

Sequência de cDNA completo:

ATGTCGGCGGCGCCGGTAGTGGACGCGGAGTACATGGCGGAGGTCGAGAGGGCG
CGCCGCGACCTGCGCGCCCTCATCGCCAGCAAGAGCTGCGCTCCCATCATGCTCCG
CCTGGCGTGGCACGACGCCGGCACGTACGACAAGGCGACGAAGACGGGAGGGCC

CAACGGCTCCATCAGGTTCCCGCAGGAGTACAGCCACGCCGCGAATGCGGGCATCA
AGATTGCTATTGATCTGCTAGAACCGATGAAACAGAAGCATCCCAAGATTACCTATGC
TGACCTGTATCAGCTTGCCGGAGTTGTTGCCGTTGAAGTCACCGGTGGGCCAACTAT
AGATTATGTTCCTGGCAGGAGGGATTCTTCAGATTCCCCAGAGGAAGGCCGTTTGCC
AGATGCTAAGAAAGGTGCTGCGCATCTGAGGGAAGTCTTCTACAGGATGGGCTTATC
AGACAAGGACATAGTAGCACTCTCAGGCGGCCATACTCTCGGGAAAGCTCGTCCCG
AAAGATCAGGATTTGATGGTGCCTGGACAAAGGATCCACTGAAATTTGACAACTCCT
ATTTCATTGAGCTTCTGAAAGAGAATTCAGAAGGGCTTCTGAAGTTGCCTACTGACAA
GGCTCTTGTTGAAGATCCTACATTCCGCCGCTATGTCGAACTATATGCCAAGGACGA
AGATGCATTTTTCAGGGACTATGCAGAGTCGCACAAGAAGCTATCTGAGCTGGGGTT
CACGCCTCCTCGCTCTGCATTCATCTACAAATCATGCCAGAAACCAAAGTCCCTGTT
GATGCAAACTGCAGCTGGGGTTGCAGTTGCTGCTGCAGTTGTCGCATGGGCTTACC
TCTGTGAATCCAACAAGAGGCTTGGCTAA

OsAPX4 – (código de acesso no NCBI: AK070842)

Sequência de cDNA completo:

ATGGCCGCCCCGGTCGTGGACGCCGAGTACCTCCGCCAGGTCGACAGGGCGCGCC
GCCACCTCCGCGCCCTCATCTCCTCCAAGGGATGCGCGCCCATCATGCTCCGCCTC
GCATGGCATGACGCGGGCACTTATGACGTGAACACAAAAACTGGTGGTGCAAATGG
TTCAATTAGATACGAGGAAGAGTACACTCACGGTTCAAATGCTGGTCTAAAGATTGCT
ATTGATCTTCTCGAGCCTATTAAAGCCAAGAGCCCTAAGATCACATATGCTGACCTTT
ATCAGCTTGCTGGAGTTGTTGCAGTTGAAGTTACTGGGGGTCCAACTGTTGAGTTCA
TTCCTGGAAGACGTGATTCGTCAGTTTGCCCCCGTGAAGGGCGTCTTCCTGATGCTA
AGAAAGGTGCACTGCACTTGAGGGACATCTTTTACCGGATGGGCTTATCAGACAAAG
ATATAGTAGCTTTATCTGGGGGTCACACTCTGGGAAGGGCACATCCTGAAAGGTCTG
GATTTGAAGGCGCATGGACTCAGGAGCCTCTGAAGTTTGACAATTCGTACTTTCTTG
AGCTACTGAAGGGGGAATCTGAGGGGCTTCTGAAGCTCCCAACTGACAAGGCATTG
TTGGAAGATCCTTCATTTAGACGCTATGTGGATCTTTATGCCAGGGATGAAGACACCT
TCTTCAAGGACTACGCTGAATCGCACAAGAAGCTTTCTGAACTTGGCTTCACTCCAC
GGAGCAGCGGCCCTGCATCTACGAAATCTGATCTTTCAACTGGTGCTGTACTCGCAC
AGAGTGCTGTCGGGGTAGCTGTTGCTGCAGCTGTAGTTATCGTGAGCTACCTATACG
AGGCTTCTAAGAAGAGCAAGTAA

OsAPX5 – (código de acesso no NCBI: AK073910)
Sequência de cDNA completo:

ATGGCCGTCGTGCACCGCATCCTCCGCCGCGGGCTCTCCGCCGCCTCTCCCCTCCC
TTCTCTCCGCGGCCTCCTCCTCGTCTCCCCTCAGGAGCTCGGCAGGCGTCCGGCGA
GCTCGTCGTCGTCGGCGGCGGCGGCTGCGGGGGACGTGGAGGCTGAGCTGCGCG
CCGCGCGGGAGGACGTCAGGCAGCTGCTCAAGTCCAACCCCTGCCATCCCATCCTG
GTTCGGTTAGGGTGGCATGACGCTGGCACTTACGATAAGAACATCACTGAATGGCCA
AAGTGTGGTGGTGCGAATGGTAGCTTGAGATTTGGAGTTGAGTTAGTACATGCAGCT
AACAAAGGTCTTCTGAAGGCGTTATTCTTGGTCATTCCCATCAAGAGCAAGTATGCAG
GTGTCACTTATGCAGATATATTTCAACTTGCCAGTGCTACAGCCATTGAGGAAGCCG
GTGGCCCCAAGATCCCCATGATCTATGGAAGGGCTGATGTTGCTGATGGTGAAGAAT
GCCCACCTGAGGGGAGACTTCCTGCTGCTGACCCACCTTCACCTGCTGAACACTTG
CGAGAAGTATTCTACAGAATGGGCCTGAGTGACAAGGAAATTGTTGCGTTGTCAGGA
GCTCATACACTTGGTCGAGCTAGACCAGAGCGTAGTGGATGGGGTAAACCAGAAAC

AAAATACACTGAAAATGGGCCTGGTGCTCCTGGAGGGCAATCTTGGACATCTGAGTG
GCTCAAGTTTGACAACAGCTACTTCAAGGAAATCAAAGAACGCCGAGATGAGGATCT
TCTAGTTCTCCCTACTGATGCTGTGCTCTTTGAGGACTCATCATTCAAGATCCATGCT
GAAAAGTATGCTGAGGATCAGGATGCATTTTTCGAAGACTATGCTGAAGCTCATGCC
AAACTGAGCAATCTTGGGGCAAAGTTTGATCCTCCAAAGGGTATTTCACTTGAATAG

OsAPX6 – (código de acesso no NCBI: AK061107)
Sequência de cDNA completo:

ATGGCCGTCGTCCACCGCCTCCTCCGCCGCGGCCTCTCCGCCGCCTCTCCCCTCCC
CTCTCTTCAGGAGCTCGGGAGGCGTCCGGCGAGCTCGTCGGCGGCGGCGGCGGG
GGACGCGGCGGCTGAGCTGCGGGGCGCGCGGGAGGACGTCAAGCAGCTGCTCAA
GTCCACCTCCTGCCATCCCATCCTGGTTCGGTTAGGGTGGCATGATGCTGGTACTTA
TGACAAGAACATTACTGAATGGCCAAAGTGTGGTGGTGCCAATGGTAGCTTGAGATT
CGAAATTGAGTTAAAACATGCGGCTAATGCAGGTCTTGTGAATGCTTTGAAGCTGATC
CAGCCCATCAAAGACAAGCATGCAGGTGTCACTTATGCAGATCTGTTTCAGCTCGCC
AGTGCTACAGCCATTGAGGAAGCCGGTGGCCCCAAGATCCCCATGATCTATGGAAG
GGTTGATGTTGCTGCCCCTGAACAATGCCCGCCAGAGGGGAGACTTCCTGCTGCTG
GCCCTCCTTCACCTGCGGAACATCTACGAGAAGTATTCTATAGAATGGGCCTGAGTG
ACAAGGAAATTGTTGCATTGTCAGGAGCTCATACACTTGGACGATCTAGACCAGAGC
GCAGTGGATGGGGCAAACCAGAAACTAAATACACTAAAAACGGACCTGGTGCACCT
GGAGGGCAATCTTGGACATCACAGTGGCTGAAGTTTGATAATAGCTACTTCAAGGAC
ATCAAAGAACGCCGAGATGAGGACCTTCTAGTTCTGCCTACTGATGCTGTGCTCTTT
GAGGACTCATCATTCAAGATCTATGCTGAAAAGTACGCCGCAGATCAGGATGCATTT
TTTGAAGACTATGCTGAAGCTCATGCCAAACTGAGCAATCTCGGAGCAAAGTTTGAT
CCTCCAAAGGGTATTTCACTGGAATAA

OsAPX7 – (código de acesso no NCBI: AK103344)

Sequência de cDNA completo:

ATGGCGGCCCAGCGACTCGCCGCCCTCCACGCCGCCGCGCCGTCGGCCTTCTCCT
CCACCTCCTCCGCCTCGCATGGCCGCCCCGCGGCGCGATCCAGCACCACCGCTCT
CCTCCCGGTGGCCCTCCCGCGCGCCTCCGCCACCCTCCGCGCCGCACCTTCTCGG
CTCCTCCCCCAGGAGGCGAAGGCGGCGGGGAGCGGCAGGTCGGTGATGTGCATGG
CGTCGGCGTCGGCGTCGGCGGCGTCGGCGGCGGTGGCGTCGGGCGCGGCGGAG
CTGAAGGCCGCTCGCGAGGACATCAGGGAGCTCCTCAAGACGACGCACTGCCACC
CCATCCTGGTTCGTCTTGGATGGCATGATTCCGGTACGTATGACAAGAATATTAAAGA
GTGGCCACAACGAGGTGGAGCTAATGGAAGCTTGAGATTTGATGTTGAGTTAAAACA
TGGAGCCAATGCTGGGCTGGTAAATGCTCTAAAGCTTGTCCAACCTATCAAGGACAA
ATACCCAAATATCTCATATGCGGATTTATTCCAGCTGGCAAGTGCTACAGCAATTGAG
GAAGCTGGTGGTCCAAAGATTCCAATGACATATGGACGGATTGATGTCACAGGTCCT
GAGCAGTGTCCACCTGAGGGGAAGCTTCCCGATGCTGGCCCAAGTGCACCTGCGGA
TCACTTGAGGAAGGTATTTTACAGGATGGGTCTTGATGACAAGGAGATTGTTGTGCT
GTCTGGAGCACATACACTTGGAAGGTCCAGACCTGAACGGAGTGGCTGGGGGAAAC
CAGAAACGAAATATACTAAGAATGGCCCTGGTGCACCTGGTGGGCAATCGTGGACA
GCTGAATGGCTTAAGTTTGATAACAGTTATTTCAAGGAGATAAAAGAGAAAAGAGATC
AGGATCTCCTGGTCTTGCCTACAGATGCTGCATTATTTGAGGACCCAACATTCAAGG
TCTATGCAGAAAAATACGCAGAGGACCAAGAAGCATTCTTTAAAGACTATGCCGGAG

CTCATGCTAAACTGAGCAATCTGGGTGCAAAATTCAATCCTCCTGAGGGATTCACGTT
GGACGGTTAA

* Sequências marcadas (cinza claro) referem-se às sequências dos oligonucleotídeos iniciadores específicos projetados para amplificar as seqências de cDNA.
** Sequências marcadas (cinza escuro) referem-se ao códon de término da tradução.

APÊNDICE 2

Sequências genômicas dos genes OsAPX7 e OsAPx8 e sequência do inserto RNAi para o silenciamento simultâneo dos genes *OsAPX7* e *OsAPX8*

OsAPX7 (LOC_Os04g35520)

Sequência genômica completa

GCGAAAAACCCCCTCGAAACCCAAGGCGGCGGGCGTGGTGGTGGACTGGACTTTTT
TCACCTCACTCCGCCTCGTCCCCGCCCCAAAGGCCACGCTCCCCTCCCTCCCACCA
ACCCACCACCAGCGTCACCCATGGCGGCCCAGCGACTCGCCGCCCTCCACGCCGC
CGCGCCGTCGGCCTTCTCCTCCACCTCCTCCGCCTCGCATGGCCGCCCCGCGGCG
CGATCCAGCACCACCGCTCTCCTCCCGGTGGCCCTCCCGCGCGCCTCCGCCACCCT
CCGCGCCGCACCTTCTCGGCTCCTCCCCCAGGTCCGTGTCGGCTCGGTCTTGGGTG
GTGGTTGGGTTGGGGTTTGGGACGCCCTGGTTGTTTGTTGTTGTTGACTCGTGCTCT
GGCTGTGTGTGTGTGATCGCAGGAGGCGAAGGCGGCGGGGAGCGGCAGGTCGGT
GATGTGCATGGCGTCGGCGTCGGCGTCGGCGGCGTCGGCGGCGGTGGCGTCGGG
CGCGGCGGAGCTGAAGGCCGCTCGCGAGGACATCAGGGAGCTCCTCAAGACGACG
CACTGCCACCCCATCCTGGTATGATCTCGTCTCGTGTACACGCACCACGCGATGCG
GCTTTGGTTGCTCCGAAACTTCTCCTTCATATCAGCATCACCATGTGGGAGATTTGAT
TCGGTAGCAGAGGAGCCATCTGGCCGATCGCCTTTGCAACCAAGGATTGCACATGA
GAACAAACTCAGCTCGGAGACAGCGATGTTATGAGTTTGTGAACACACTGAATTTTCT
CTCTGGAGACAGTGATGTCATGAATCTGTGAACACACTGAATATGCTCTCTGGAGCA
ATGCTGGAATTTAACATCACGAGGAAGTAATTTCATGACCAGCTTGCCTCTCAAATAG
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OsAPX8 (LOC_Os02g3410)

Sequência genômica completa

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Inserto RNAiOsAPX7/8 (238 pb)

CTCTAAAGCTTGTCCAACCTATCAAGGACAAATACCCAAATATCTCATATGCGGATTT
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TTCCCGATGCTGGCCCAAGTGCACCTGCGGATCACTTGAGGAAGGTATTTTACAGGA
TGGGTCTTGA

* Sequências marcadas referem-se as sequências dos oligonucleotídeos iniciadores específicos para a construção RNAiOsAPX7/8.

APÊNDICE 3

Tabela mostrando as similaridades entre a sequência RNAi e os genes de *OsAPX*

Similaridades entre a sequência da construção RNAi e os genes de *OsAPX.*

Construção RNAi	*OsAPX1*	*OsAPX2*	*OsAPX3*	*OsAPX4*	*OsAPX5*	*OsAPX6*	*OsAPX7*	*OsAPX8*
RNAiOsAPX7/8 (238 nt)	55% (8 nt)	58% (9 nt)	55% (11 nt)	59% (9 nt)	70% (13 nt)	71% (11 nt)	100% (290 nt)	81% (34 nt)

Nota: O número de nucleotídeos compartilhados em sequência contígua entre cada um dos genes de *OsAPX* e a sequência alvo da construção RNAi são indicados entre parênteses.