UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

DEPARTAMENTO DE GENÉTICA

PROGRAMA DE PÓS-GRADUAÇÂO EM GENÉTICA E BIOLOGIA MOLECULAR

CARACTERIZAÇÃO DE MECANISMOS ENVOLVIDOS COM A HOMEOSTASE DE ESPÉCIES REATIVAS DE OXIGÊNIO NA RESPOSTA DE PLANTAS A DIFERENTES ESTRESSES

Tese de Doutorado

DOUGLAS JARDIM MESSEDER DE ALVARENGA

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DOUGLAS JARDIM MESSEDER DE ALVARENGA

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

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SUMÁRIO

RESUMO

As espécies reativas de oxigênio (ROS) são importantes moléculas sinalizadoras, ou mensageiros secundários, em uma complexa rede de sinalização, que em plantas, é fundamental para o desenvolvimento, e para a resposta a diferentes estímulos ambientais. Por outro lado, estas moléculas representam uma ameaça oxidativa à celula, e em altas concentrações podem danificar diferentes componentes celulares. Dessa forma, as vias de produção e eliminação de ROS devem ser finamente moduladas. Apesar destas vias terem sido amplamente estudadas, incontáveis aspectos ainda permanecem desconhecidos. Este trabalho objetivou estudar os mecanismos de geração e eliminação de *ROS* nas mitocôndrias e cloroplastos, e seus efeitos no desenvolvimento e na resposta de plantas ao estresse.

Assim, foi demonstrado que a enzima succinato desidrogenase (SDH), correspondente ao complexo II da respiração, é um importante sítio de geração de ROS em células vegetais. Além disso, a manipulação da geração de ROS em organelas, como a mitocôndria e o cloroplasto, promoveu alterações claras no padrão de desenvolvimento e nas respostas de plantas a diferentes estresses. Enquanto a indução da geração mitocondrial de ROS via SDH inibiu o desenvolvimento e levou a ativação da expressão de genes de defesa. Por outro lado, alterações nas respostas antioxidantes no cloroplasto, via manipulação genética das ascorbato peroxidases cloroplastídicas (OsAPX7 e OsAPX8), embora tenham afetado em menor grau o desenvolvimento da planta, modularam os parâmetros fisiológicos e a resposta ao estresse. Desta forma, plantas de arroz silenciadas, ou nocaute, para a isoformas cloroplastídicas de APX apresentaram um padrão diferenciado de abertura estomática e tolerância ao estresse hídrico. Além disso, experimentos de monohíbrido permitiram a identificação dos fatores de trasncrição OsDST, OsABF7, Os11g28270 e OsVOZ1, como potenciais reguladores da expressão de *OsAPX8*.

A complexidade das respostas induzidas por ROS indicam que estas possuem uma alta especificidade e dependem da localização subcelular e da atividade de cada um dos componentes dessa intricada rede de sinalização, assim como o nível de expressão de cada um deles. O conjunto dos resultados obtidos amplia a visão do papel das ROS no desenvolvimento vegetal e nos mecanismos de respostas de plantas a estímulos ambientais geradores de estresse oxidativo

ABSTRACT

The reactive oxygen species (ROS) are important signaling molecules, or secondary messengers, involved in a complex signaling network, which, in plants, is essential for development, and different environmental stimuli responses. Moreover, these molecules represent an oxidative risk to the cell, and at high concentrations may damage various cellular components. Thus, the ROS production and elimination routes should be finely regulated. Although these pathways have been extensively studied, many aspects remain unknown. Here we investigated the mechanisms of generation and elimination of ROS in mitochondria and chloroplasts, and its effects in plant development and stress responses.

The results demonstrated that the enzyme succinate dehydrogenase, which corresponds to the mitochondrial complex II, is an important site of ROS production in plant cells. In addition, the control of ROS production in cellular organelles, such as mitochondria and chloroplast, promotes changes in development patterns and in plant stress response. While the induction of mitochondrial ROS production by SDH inhibit the development and activated defense genes expression, changes in chloroplast antioxidant response, by genetic manipulation of chloroplastic ascorbate peroxidases (OsAPX7 e OsAPX8), modulates physiologic parameters and stress response, despite inducing lower changes related to plant development. In this way, rice plants silenced or knockout for chloroplastic isoforms of APX showed a differential stomata opening pattern and drought stress tolerance. In addition, one-hybrid experiments, allowed the identification of the transcription factors OsDST, OsABF7, Os11g28270 e OsVOZ1, as potential regulators of the *OsAPX8* expression.

The complexity of the responses induced by ROS indicates that these mechanisms have a high specificity and is dependent of the subcelullar location, their activity, and the expression of each one of the components of this signaling network. The results obtained expands our vision of the role of ROS in plant development and in plant responses to environmental stimuli related to oxidative stress.

1- INTRODUÇÃO GERAL

1.1 Espécies Reativas de Oxigênio

O oxigênio molecular se tornou abundante na atmosfera terrestre há bilhões de anos e sua presença permitiu o surgimento de organismos aeróbios, capazes de utiliza-lo como aceptor final de elétron em suas reações de oxi-redução. Esse processo deu origem à respiração, a qual proporcionou um rendimento energético muito superior ao da fermentação, que até então era a principal via de transdução de energia presente nos seres vivos. No entanto, devido a alta eletronegatividade dos átomos de oxigênio, e a sua capacidade de comportar elétrons, durante as reações de oxi-redução do seres vivos inevitavelmente ocorre a formação de espécies reativas de oxigênio (*ROS,* do inglês *Reactive Oxygen Species*), que são tidas como subprodutos dessas reações. As *ROS*, tais como o radical superóxido ($O_2^{\cdot -}$), peróxido de hidrogênio (H_2O_2), radical hidroxil ($HO^{\cdot}$) e oxigênio singleto ($^1O_2$), em geral são formadas por sucessivas adições de elétrons ao oxigênio molecular ou como resultado de sua excitação.

O ânion superóxido é resultante da redução monovalente do oxigênio molecular, que passa a obter carga negativa e torna-se uma espécie radicalar. Como tal, o superóxido possui natureza polar e não é capaz de atravessar membranas facilmente. Já, o peróxido de hidrogênio possui natureza apolar e, portanto, atravessa facilmente as membranas biológicas. O peróxido de hidrogénio não possui, por si só, muita reatividade, mas é capaz de dar origem ao radical hidroxil, que além de ser uma espécie radicalar, é a mais reativa de todas as formas de *ROS* apresentadas, possuindo o menor tempo de meia vida. Já o oxigênio singleto, é resultante da excitação da molécula de oxigênio por energia luminosa, em uma reação conhecida como fotossensibilização. Ao contrário das demais formas de *ROS*, o oxigênio singleto não é resultante do processo de ionização da molécula de oxigênio.

Devido à inevitável geração de *ROS*, para se adaptarem à condição aeróbica, os seres vivos tiveram que desenvolver diferentes mecanismos para lidar com a presença destas moléculas, inclusive aproveitando de sua alta reatividade em processos fisiológicos. De fato, atualmente os mecanismos de geração de *ROS* têm uma importante função sinalizadora em diferentes seres vivos. Em plantas, as *ROS* podem atuar como segundo mensageiro em diferentes processos fisiológicos, como crescimento, ciclo celular, desenvolvimento, senescência, morte celular programada, condutância estomatal, sinalização hormonal, regulação da expressão gênica, e nas respostas celulares às condições ambientais e a diferentes estresses bióticos e abióticos, tais como alta luminosidade, frio, calor, seca, inundação, ataque de patógenos, etc. (KOVTUN *et al.*, 2000; NEILL *et al.*, 2002; FOYER & NOCTOR, 2005; SLESAK *et al.*, 2007; STONEBLOOM *et al.*, 2009).

Por serem moléculas altamente reativas, em altas concentrações as *ROS* são consideradas tóxicas, pois são capazes de causar danos oxidativos em diferentes componentes celulares, tais como proteínas, lipídios e DNA, podendo induzir, inclusive, morte celular. Desta forma, os mecanismos de geração e eliminação de *ROS* devem ser finamente modulados. A situação em que ocorre um desbalanço entre a produção de *ROS* e os mecanismos de defesa antioxidante é chamada de estresse oxidativo, a qual corresponde a um fator central para o fenótipo de plantas submetidas a estresses bióticos e abióticos (MITTLER *et al.*, 2004).

1.2 Geração de *ROS* em Células Vegetais

É estimado que entre 1 a 3 % de todo o oxigênio consumido por tecidos vegetais seja convertido em *ROS* (PUNTARULO *et al.*, 1988). Dados na literatura demonstram que em células vegetais, as *ROS* são produzidas primariamente pelo cloroplasto, pelo peroxissomo e pela mitocôndria (CHANCE *et al.*, 1979; FOYER *et al.*, 1994; SLESAK *et al.*, 2007).

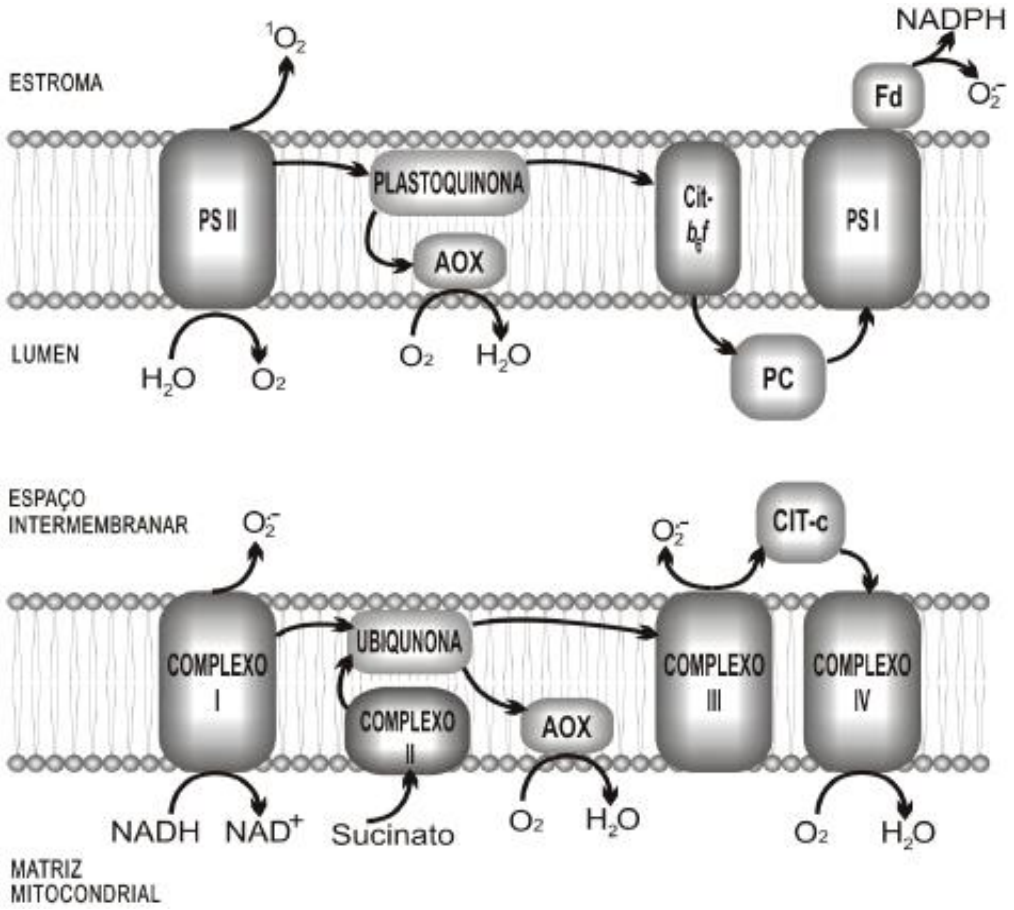
O cloroplasto, onde ocorre grande absorção de energia luminosa, é um importante sítio de produção de oxigênio singleto e anion superóxido. A

formação de oxigênio singleto ocorre principalmente no fotossistema II (PS-II), através da transferência da energia não dissipada e armazenada nas moléculas de clorofila para a molécula de oxigênio (BHATTACHARJEE, 2010). Durante a fotossíntese também ocorre a formação de superóxido, que é gerado majoritariamente pela “Reação de Menler”, através da transferência de elétron da ferrodoxina reduzida para o oxigênio molecular (FOYER & SHIGEOKA, 2011; FOYER et al., 2012). Embora os cloroplastos tenham um importante papel na geração de *ROS*, em tecidos fotosintetizantes, os peroxissomos são considerados os principais sítios intracelulares de geração de *ROS*. No peroxissomo a produção de peróxido de hidrogênio ocorre através da fotorrespiração, como subproduto da oxidação da molécula de glicolato (KARUPPANAPANDIAN et al., 2011; SHARMA et al., 2012).

É estimado que em folhas adultas, a produção de peróxido de hidrogênio nos peroxissomos e clorosplastos pode ser de 30 a 100 vezes maior do que nas mitocôndrias (FOYER & NOCTOR, 2003; BHATTACHARJEE, 2010). Por outro lado, em tecidos não fotosintetizantes, a geração de *ROS* ocorre principalmente na mitocôndria (PUNTARULO *et al.*, 1991), onde o oxigênio molecular é capaz de interagir com as formas reduzidas de alguns componentes da cadeia transportadora de elétrons (CTE), como flavinas e ubiquinona, sendo reduzido monovalentemente ao ânion superóxido, que posteriormente é dismutado em peróxido de hidrogênio (MØLLER, 2001). As cadeias transportadoras de eletrons cloroplastídica e mitocondrial, bem como seus respectivos sítios de geração de *ROS* estão indicados na figura 1.

Classicamente é descrito que os principais sítios de geração de *ROS* na CTE são o complexo mitocondrial I, através do transporte reverso de elétrons (CHANCE *et al.*, 1979; TURRENS & BOVERIS, 1980), e o complexo III, através da formação do radical ubisemiquinona (TURRENS *et al.*, 1985). No entanto, a análise estrutural do complexo II, correspondente à enzima succinato desidrogenase (SDH), sugere que esta enzima é capaz de reduzir monovalentemente a molécula de oxigênio, formando superóxido (YANKOVSKAYA et al., 2003). De fato, foi demonstrado que a SDH seria um importante sítio direto da geração de *ROS* em mamíferos (QUINLAN et al., 2012). No entanto, em plantas, a contribuição direta da SDH na produção

mitocondrial de *ROS* ainda não foi demonstrada, embora trabalhos em tomate (*Solanum lycopersicum*) e *Arabidopsis thaliana* demonstrem que mutações ou o silenciamento dos genes de SDH levam a alterações no estado redox das plantas, decorrentes da diminuição do conteúdo líquido de peróxido de hidrogênio. Além disso, essas plantas apresentam ainda um padrão alterado de alguns processos fisiológicos, tais como fotossíntese, produção de pólen e resposta à estresse biótico (ARAÚJO et al., 2011; LEÓN et al., 2007; FUENTES et al., 2011).



Retirado de BARBOSA *et al.*, 2014

Figura 1: Formação de ROS nas cadeias transportadoras de eletrons cloroplastídica e mitocondrial. *(a) Na cadeia transportadora de elétrons cloroplastídica, ocorre a oxidaçao da molécula de água em oxigênio molecular, por meio da excitação luminosa da clorofila presente no fotossitema II (PSII). O eletron excitado proveniente dessa redução é transportado por meio da*

plastoquinona, do citocromo b_6f (cit-b_6f) e da plastocianina (PC), chegando ao fotossistema I (PSI), onde é novamente excitado e transferido para molécula de NADP+ via ferrodoxina (Fd). No cloroplasto a formação de oxigênio singleto ocorre prodominantemente no PS-II e a de superóxido ocorre no PS-I. (b) Na cadeia transportadora de elétrons mitocondrial, o aceptor final do elétron é o oxigênio molecular, que é reduzido à agua via citocromo c oxidase (complexo IV). Este eletron é proveniente da oxidação da moléculas de NADH (via complexo I) e succinato (via complexo II), e será transportado por meio da molécula da ubiquinona, do complexo III, e do citocromo C (cit. C), chegando finalmente no complexo IV onde será transferido ao oxigênio, formando água. Na respiração a formação de superóxido ocorre nos complexos I e III. Em ambas as cadeias apresentadas o pool de quinonas reduzidas (plastoquinona ou ubiquinona), pode ser controlado por meio da atividade de oxidases alternativas (AOX).

Em plantas, as *ROS* podem ser produzidas ainda através de outros processos fisiológicos, como a β-oxidação de ácidos graxos nos glioxissomos, a ação da NADPH oxidase na membrana plasmática, ou por diferentes enzimas na matriz extracelular (MYLONA & POLIDOROS, 2010). O citosol não é considerado uma grande fonte de *ROS*, no entanto atua como um depósito para moléculas produzidas por outros compartimentos subcelulares

1.3 Produção e eliminação de *ROS* como mecanismos de transdução de sinais

Os níveis de *ROS* nos diferentes compartimentos celulares é determinado pela interação entre múltiplos mecanismos de produção e eliminação de *ROS*. Estes mecanismos são controlados através de vias de transdução de sinais que constituem o "ciclo básico de *ROS*" (RASOOL *et al*., 2013). Durante o ciclo de vida dos organismos, tais vias são capazes de monitorar os níveis de *ROS* através do controle da expressão gênica e da

atividade de diferentes enzimas ou rotas metabólicas que participam da homeostase redox (figura 2)

Em condições de estresse, há um desequilíbrio da homeostase celular, o que resulta no aumento da produção de *ROS*. As *ROS* estão envolvidas em diferentes vias de transdução de sinal em resposta ao estresse, e portanto, são essenciais para a indução de vias de defesa. De fato, diferentes mecanismos de sinalização mediados por *ROS* vêm sendo propostos, e diferentes receptores vêm sendo descritos, como canais iônicos, histidinas kinases e fosfatases (APEL & HIRT, 2004; ASADA, 2006; DEMIDCHIK & MAATHUIS, 2007; FOYER & NOCTOR, 2009; SIERLA *et al*., 2013; DEMIDCHIK *et al*., 2014).

A modulação da atividade de canais iônicos é considerado o mais rápido e eficiente mecanismo de resposta a fatores internos e externos, e envolve principalmente dois mecanismos: a mudança da composição iônica, o que pode regular a atividade de enzimas e a pressão osmótica, e a mudança do potencial elétrico através de biomembranas, o que pode modular a atividade de diferentes transportadores e enzimas associadas (DEMIDCHIK et al., 2015). Os mecanismos mediados pela modulação de canais iônicos, incluem por exemplo, o fechamento estomático induzidos por fitohormônios, como ácido abscísico (ABA) ou jasmonato, em resposta a estresse hídrico (MUNEMASA *et al*., 2011). As *ROS* também são capazes de modular a atividade de outras enzimas regulatórias, em especial, kinases e fostatases, como MAP kinases, Ser/Thr kinases, MAPK phosphatases, etc. No entanto, o mecanismo exato pelo qual esta regulação ocorre ainda não está totalmente descrito (VAN BREUSEGEM *et al*., 2008, PITZSCHKE & HIRT, 2009; RODRIGUEZ *et al*., 2010).

Diferentes fatores de transcrição também podem atuar com receptores do sinal redox. Por exemplo, o fator de transcrição TGA1 em *Arabidopsis thaliana*, que regula a expressão de NPR1, uma proteína de resistência à doença, possui dois resíduos de histidina específicos e a oxidação destes resíduos leva à perda da capacidade do fator de transcrição em se ligar ao DNA (DESPRES *et al*., 2003). Os fatores de transcrição do tipo *Heat shock* também estão envolvidos na sinalização redox, sendo ativados durante o

estresse e regulando diferentes genes relacionados a mecanismos de defesa (MILLER & MITTLER, 2006; HONG *et al*., 2013).

Apesar da importância das *ROS* nas vias de transdução de sinais em resposta ao estresse, o aumento da produção de *ROS* pode representar uma ameaça oxidativa para a célula, caso não haja um fino controle da homeostase redox. Desta forma, em muitas condições de estresse, ocorre também a indução de sistemas antioxidantes, o que colabora para a prevenção de danos oxidativos.

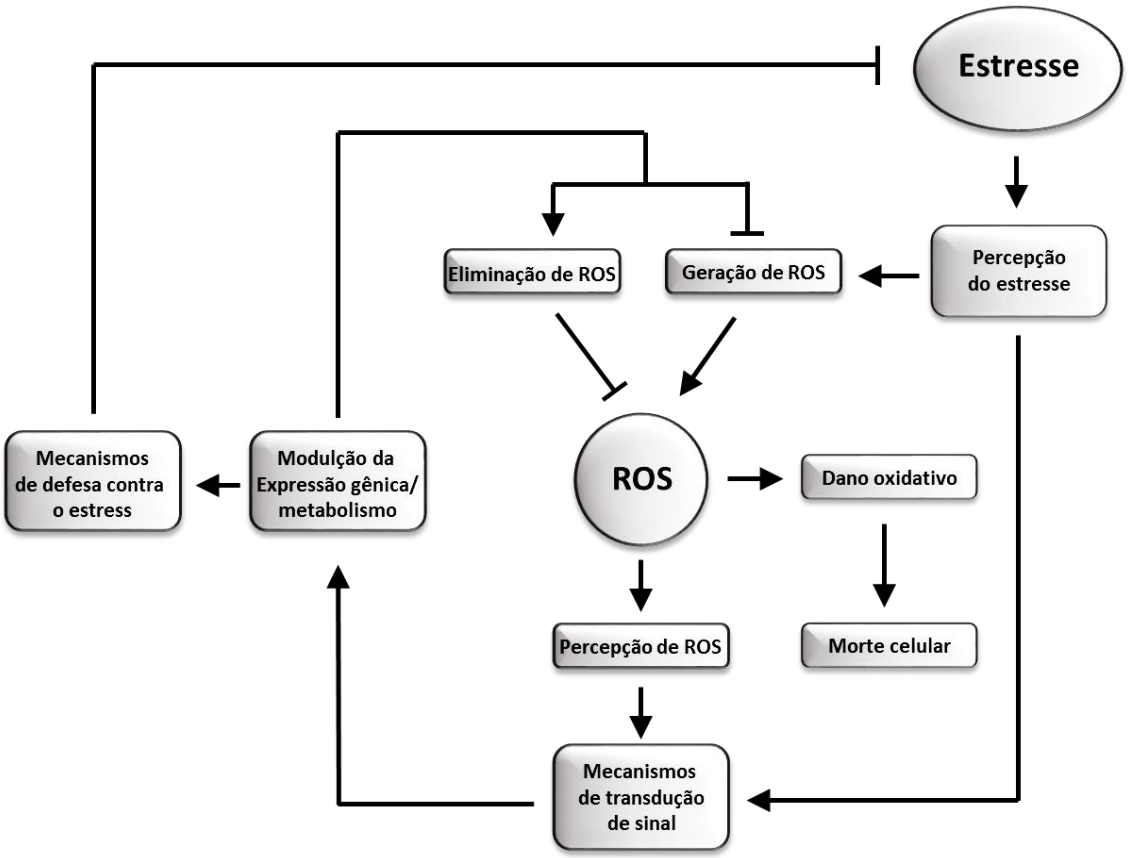


Figura 2: "Ciclo básico de ROS" em células vegetais. *Este ciclo regula os níveis celulares de ROS durante o metabolism normal e nas respostas a estresses bióticos e abióticos.*

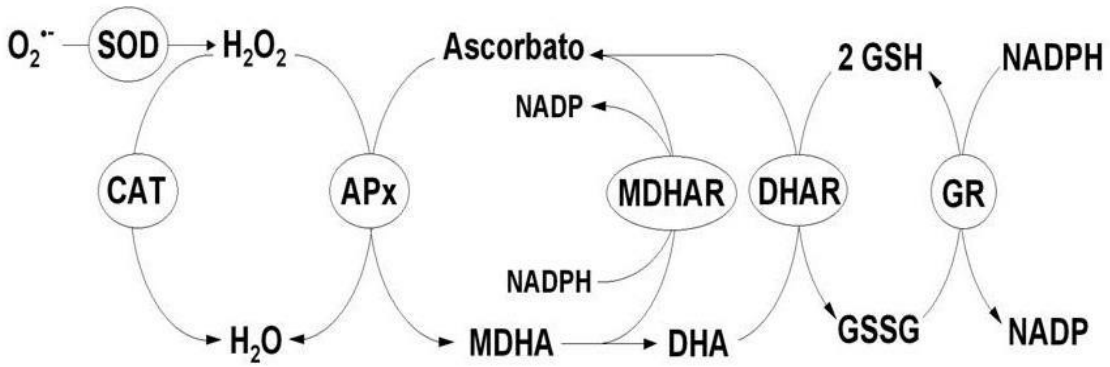
1.4 Estratégias de Defesa Contra o Estresse Oxidativo em Células Vegetais

O papel das *ROS* no metabolismo celular, como reguladoras de processos fisiológicos ou como produtos tóxicos, é dependente da sua concentração intracelular (FOYER & NOCTOR, 2005). Desta forma, os seres vivos desenvolveram, ao longo da evolução, diferentes mecanismos de defesa antioxidante que os permitem, controlar os níveis celulares de *ROS*. Em plantas, existem sistemas enzimáticos e não enzimáticos de controle dos níveis de *ROS* e que atuam em diferentes compartimentos subcelulares.

Os componentes não enzimáticos da defesa antioxidante incluem moléculas como o ascorbato (*AsA*), glutationa reduzida (GSH), tocoferol, carotenoides, flavonoides e componentes fenólicos, que reagem diretamente, neutralizando as *ROS* e prevenindo a ocorrência de danos aos componentes celulares (MITTLER *et al.*, 2004; GRATÃO *et al.*, 2005; SCANDALIOS, 2005). Já o sistema enzimático de detoxificação de *ROS* em células vegetais inclui diferentes enzimas, presentes em diferentes compartimentos subcelulares, entre elas a superóxido dismutase (SOD), a catalase (CAT), a glutationa peroxidase (GPX) e a ascorbato peroxidase (APX). Essas enzimas atuam coordenadamente na eliminação de *ROS* para manter a homeostase redox dos diferentes compartimentos celulares. A enzima SOD constitui a primeira linha de defesa convertendo o radical superóxido em peróxido de hidrogênio, o qual é detoxificado pela atividade da CAT, e também pela ação de diferentes peroxidases, que requerem a redução de substratos específicos como doadores de elétrons. Nesta última categoria estão incluídas as APXs, dependentes de ascorbato, e as GPXs, que dependem de glutationa reduzida (NOCTOR & FOYER, 1998).

Em plantas, a APX corresponde a uma das mais importante família de peroxidase envolvida na remoção intracelular de peróxido de hidrogênio. APX catalisa a primeira etapa do ciclo ascorbato-glutationa, a principal rota antioxidante em células vegetais (Figura 3). Neste ciclo, o ascorbato e a glutationa são empregados como substrato redutor para detoxificar o peróxido de hidrogênio, e posteriormente são reciclados através da oxidação de

NAD(P)H. Além da APX, a monodehidroascorbato redutase (MDHAR), a dehidroascorbato redutase (DHAR), e a glutationa redutase (GR), também catalisam importantes etapas desta rota. O principal papel do ciclo ascorbato-glutationa é a proteção contra danos oxidativos principalmente no cloroplasto, embora ele também atue no citoplasma (NOCTOR & FOYER, 1998).



Retirado de TEIXEIRA et al., 2004

Figura 3: Eliminação de ROS via Ciclo Ascorbato-Glutationa. *APX – Ascorbato peroxidase; CAT – Catalase; DHA – Dehidroascorbato; DHAR – Dehidroascorbato redutase; MDHA– Monodehidroascorbato; MDHAR – Monodehidroascorbato-redutase; GR – Glutationa redutase; GSH – Glutationa reduzida; GSSG – Glutationa oxidada; SOD – Superóxido dismutase*.

1.5 Papel da Ascorbato Peroxidase no metabolismo antioxidante

Em plantas, a maioria das enzimas antioxidantes são codificadas por famílias multigênicas. Isso confere uma grande vantagem adaptativa, permitindo uma regulação diferencial de cada membro da família gênica em resposta a estímulos endógenos (diferentes tecidos e estágios do desenvolvimento) e exógenos (por exemplo, estímulos ambientais). Diferentes isoformas de APX estão presentes nas diversas espécies. Eucalipto (*Eucaliptus grandis*) e tomateiro, por exemplo, possuem seis isoformas de APX, enquanto o feijoeiro comum (*Phaseolus vulgaris*) conta com oito isoformas e *Arabidopsis thaliana* nove; por outro lado em espinafre (*Spinacia oleracea*) apenas quatro genes codificam as diferentes isoformas de APXs. Em arroz (*Oryza sativa*), a família de genes de APX é constituída por 8 membros denominados de *OsAPX1* a *OsAPX8*. A localização subcelular predita de cada isoforma de APX em arroz,

baseada na presença de peptídeos sinais adicionais, é descrita a seguir: Os genes *OsAPX1* (cromossomo 3) e *OsAPX2* (cromossomo 7) codificam isoformas citosólicas; *OsAPX3* (cromossomo 4) e *OsAPX4* (cromossomo 8) codificam isoformas peroxissomais; as isoformas mitocondriais são codificadas pelos genes *OsAPX5* (cromossomo 12) e *OsAPx6* (cromossomo 12), enquanto *OsAPX7* (cromossomo 4) e *OsAPX8* (cromossomo 2) codificam isoformas cloroplastídicas, sendo a primeira, solúvel no estroma, e a segunda, ligada à membrana tilacoidal. É relevante relatar o alto grau de conservação na estrutura gênica entre genes de OsAPX que codificam isoformas de um mesmo compartimento subcelular (TEIXEIRA *et al*., 2004).

O arroz é uma das principais plantas modelos para estudos biológicos. E isso se dá principalmente por dois fatores: O arroz apresenta o menor genoma entre os cereais (OUYANG *et al*., 2007) e apresenta sintenia na estrutura e organização do genoma, com outros membros da família Poaceae (MOORE *et al*., 1995), permitindo assim, que os resultados obtidos para o arroz possam ser usados como referência para outras espécies (PATERSON *et al*. , 2005). Além disso, o arroz possui uma importância sócio/econômica, uma vez que entre os cereais, é o alimento mais cultivado no mundo, sendo essencial para mais de 2,4 bilhões de pessoas (EMBRAPA, 2015).

Diferentes estudos demonstraram que as diferentes isoformas de APX em arroz apresentam padrões distintos de expressão, estando envolvidas com a regulação fina dos níveis de peróxido de hidrogênio nos diferentes compartimentos subcelulares em decorrência do estímulo ambiental (MENEZES-BENAVENTE *et al*., 2004; TEIXEIRA *et al*., 2004; 2006; ROSA *et al*., 2010; BONIFACIO *et al*., 2011; LAZZAROTTO *et al*., 2011; CAVERZAN *et al*., 2014). Estas análises levantam muitas questões sobre o papel desempenhado pelas diferentes isoformas APX no metabolismo antioxidante em células vegetais.

Por meio de estudo de genética reversa, usando a tecnologia de RNA de interferência (RNAi), o papel desempenhado pelas diferentes isorformas de APX em arroz no metabolismo antioxidante e na resposta ao estresse vem sendo elucidado. Trabalhos anteriores demonstraram que o duplo silenciamento da

isoformas citosólicas de APX (*OsAPX1* e *OsAPX2*), leva ao aumento da sensibilidade ao estresse por alumínio, além induzir a regulação da expressão de proteínas relacionadas com vias fotoquímicas, ciclo de Calvin e fotorespiração. Por outro lado, as plantas silenciadas individualmente para *OsAPX1* e *OsAPX2* apresentam problemas no desenvolvimento (ROSA *et al*., 2010, BONIFACIO *et al*., 2011). Já plantas silenciadas para *OsAPX3* e *OsAPX4* apresentam alterações no padrão de senescência (dados não publicados). Em relação às isoformas mitocondriais de APX em arroz (*OsAPX5* e *OsAPX6*), calos transgênicos simultaneamente silenciados para essas isoformas não regeneraram plantas, indicando que as APXs mitocondriais poderiam exercer um papel essencial para o desenvolvimento vegetal (dados não publicados).

Quanto às isoformas cloroplastídicas de APX (*OsAPX7* e *OsAPX8*), foi demonstrado que em diferentes situações de estresse, como alta luminosidase, frio e seca, estas isoformas possuem a expressão modulada de forma antagônica: a expressão de *OsAPX7* é geralmente induzida, enquanto a expressão de *OsAPX8* é modulada negativamente (ROSA et al., 2010; CAVERZAN et al., 2014). Além disso, foi demonstrado que o silenciamento duplo destas isoformas não acarreta mudanças fenotípicas na planta sob condições normais de crescimento, embora sob condições de estresses abióticos, as plantas de arroz silenciadas para os ambos os genes cloroplastídicos de APX sofrem fortes alterações fotossintéticas e bioquímicas, indicando que rotas fotossintéticas e do metabolismo oxidativo foram afetadas pelo silenciamento (CAVERZAN *et al*., 2014). Vale ressaltar que a modulação antagônica da expressão de *OsAPX7* e *OsAPX8* que ocorre naturalmente em resposta a estresses abióticos pode ter dificultado a interpretação do duplo silenciamento destes genes, e justificar a ausência de diferenças fenotípicas em plantas crescidas sob condições controle. Além disso, o papel individual de cada isoforma cloroplastídica de APX em arroz permanece desconhecido.

O papel funcional das isoformas cloroplastídicas de APX vem sendo estudado também em outras espécies. Em algodão (*Gossypium hirsutum*), a superexpressão de APX cloroplastídica induz um aumento na tolerância ao frio (PAYTON *et al*., 2001). Similarmente, a superexpressão da isoforma tilacoidal de APX em tabaco (*Nicotiana toabacum*) também leva a um aumento da

tolerância ao frio, bem como a estresses induzido por metil viologen (YABUTA *et al*., 2002). Já em trigo (*Triticum aestivum L.*), o silenciamento de APX tilacoidal leva à redução da atividade fotossintética e do crescimento (DANNA *et al*., 2003). Estes dados confirmam o importante papel desempenhado por APX no controle da homeostase redox e da resposta ao estresse em diferentes espécies vegetais.

1.6 Estresse oxidativo como um fator limitante na produção agrícola

Vários fatores ambientais, tais como estresses bióticos e abióticos, podem ser considerados fatores limitantes para o crescimento e a produtividade das diferentes culturas, desta forma, a escassez de áreas para expansão da agricultura já é um dos grandes desafios deste século (JACQUEMIN *et al*., 2013). Ao longo dos anos, a disponibilidade de terra arável vem diminuindo, principalmente devido a mudanças climáticas e técnicas de gestão insustentáveis, que intensificaram problemas como a erosão e a degradação do solo (STOCKING, 2003).

De acordo com a Organização das Nações Unidas para Alimentação e Agricultura (*FAO*, do inglês *Food and Agriculture Organization*), em 2050 a população mundial deverá chegar a pelo menos 9 bilhões de pessoas, e para atingir esta demanda, a produção de alimentos deverá crescer pelo menos 60% (Jacquemin *et al*., 2013). Desta forma, em um contexto de crescente demanda mundial de alimentos, desde o início do século vem se estabelecendo uma crescente necessidade da melhoria do cultivo, seja pela expansão de áreas destinadas à agropecuária, seja pelo desenvolvimento de estratégias que aumentem a tolerância de plantas a estresses ambientais (TAKEDA & MATSUOKA, 2008). Neste contexto, o estudo integrado de transcriptomas, proteômica e metabolômica vem proporcionando, ao longo dos anos, uma melhor compreensão das diferentes rotas de sinalização envolvidas na resposta de plantas ao estresse (CRAMER et al., 2011). No entanto, a compreensão dos mecanismos subjacentes às respostas de plantas entre as várias pressões ambientais ainda está longe de ser elucidado. Tendo em vista a importância do metabolismo antioxidante na manutenção dos níveis de *ROS*, e a importância

destas nos diferentes mecanismos de respostas das plantas ao estresse, é de grande importância à identificação dos mecanismos modulatórios da expressão e da atividade de enzimas que atuam na homeostase redox. Dessa forma, os genes e proteínas envolvidos no metabolismo antioxidante são alvos interessantes para o melhoramento genético de plantas para características agronômicas, tais como maior produção e menores perdas causadas por condições ambientais desfavoráveis.

2 OBJETIVOS GERAIS

Objetivo geral: Estudar os mecanismos de geração e eliminação de *ROS* em organelas importantes para o balanço redox (mitocôndria e cloroplasto), e seus efeitos no desenvolvimento e na resposta de plantas ao estresse.

Objetivos específicos:

- Determinar novos sítios de geração de *ROS*, notadamente a enzima succinato desidrogenase, conhecida como complexo II mitocondrial;

- Determinar os efeitos moleculares e fisiológicos da geração de *ROS* pela succinato desidrogenase;

- Caracterizar do ponto de vista evolutivo a succinato desidrogenase em diferentes grupos taxonômicos;

- Determinar os efeitos moleculares e fisiológicos da alteração no estado redox no cloroplasto pelo silenciamento das APX cloroplastídicas, APX7 e APX8;

- Identificar reguladores da expressão de APX8 e seu papel em vias de transdução de sinal.

3.1 CAPÍTULO 1

SUCCINATO DESIDROGENASE (COMPLEXO II MITOCONDRIAL) É UM SÍTIO DE GERAÇÂO DE ESPÉCIES REATIVAS DE OXIGÊNIO EM MITOCÔNDRIAS DE PLANTAS, REGULANDO O DESENVOLVIMENTO E A RESPOSTA AO ESTRESSE

Este capítulo é referente ao artigo “Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses”, publicado em 2015 na revista New Phytologist. Neste trabalho identificamos a enzima succinato desidrogenase (SDH) como um novo e importante sítio de geração de *ROS* em células vegetais. De fato, diferentes trabalhos vêm demonstrando que mutações em subunidades da SDH afetam diretamente a homeostase redox da célula, no entanto até o desenvolvimento deste projeto essa hipótese ainda não havia sido comprovada. Os resultados aqui apresentados demonstram ainda que a capacidade da SDH em gerar *ROS* pode ser regulada por diferentes moléculas, como o ácido salicílico e óxido nítrico (NO). Além disso, este mecanismo é fundamental na regulação da expressão gênica, controlando o desenvolvimento vegetal e as respostas de plantas a diferentes estresses.

New Phytologist

Succinate dehydrogenase (mitochondrial complex II) is a source of reactive oxygen species in plants and regulates development and stress responses

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Summary

• Reactive oxygen species (ROS) are signaling molecules that regulate plant development and responses to stresses. Mitochondria are the source of most ROS in heterotrophic cells, and mitochondrial complex I and complex III are regarded as the main sites of ROS production in plant mitochondria. Recent studies have demonstrated that succinate dehydrogenase (SDH) also contributes to mitochondrial ROS production. However, the ability of SDH to generate ROS in plants is unclear. The aim of this study was to evaluate the role of SDH in mitochondrial ROS production.

• Our results demonstrated that SDH is a direct source of ROS in *Arabidopsis thaliana* and *Oryza sativa*, and the induction of ROS production by specific SDH inhibitors impaired plant growth. In addition, this effect was accompanied by the down-regulation of cell cycle genes and the up-regulation of stress-related genes.

• However, the partial inhibition of SDH by a competitive inhibitor decreased ROS production, which was associated with increased shoot and root growth, and prevented the down-regulation of cell cycle genes and the induction of stress-related genes by noncompetitive inhibitors.

• In conclusion, SDH plays an important role in ROS production, being a direct source of ROS in plant mitochondria and regulating plant development and stress responses.

Introduction

Succinate dehydrogenase (SDH; succinate: ubiquinone oxidoreductase; mitochondrial complex II) plays a central role in mitochondrial metabolism, catalyzing the oxidation of succinate to fumarate and the reduction of ubiquinone (UQ) to ubiquinol (UQH_2), thereby linking the tricarboxylic acid (TCA) cycle and the electron transport system (ETS). Classically, SDH is composed of four subunits, named SDHA–SDHD in *Escherichia coli* and animals and SDH1–SDH4 in yeast and plants. SDHA/SDH1 is a flavoprotein subunit that has a dicarboxylate (succinate) binding site and a flavin adenine dinucleotide (FAD) cofactor. SDHB/SDH2 is an iron–sulfur protein subunit that contains three Fe–S clusters. The two other hydrophobic, membrane-anchored subunits, SDHC/SDH3 and SDHD/SDH4, contain the UQ binding site (Q-site) (Yankovskaya *et al.*, 2003).

In eukaryotic cells, the ETS drives the majority of ATP synthesis, but it is also a major source of reactive oxygen species (ROS). Complex I (NADH: ubiquinone oxidoreductase) and complex III (UQH_2: cytochrome c oxidoreductase; cytochrome bc1 complex) are generally regarded as the main sources of ROS production (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010; Møller & Sweetlove, 2010). Nevertheless, structural analyses of SDH suggested that this enzyme can produce ROS at the FAD-binding site through the monovalent electron reduction of O_2 (Yankovskaya *et al.*, 2003).

Recently, SDH was unequivocally demonstrated to be an important and direct source of ROS in mammals (Quinlan *et al.*, 2012). In addition, SDH has been recognized as an indirect modulator of superoxide production by complexes I and III (Boveris *et al.*, 1972; Møller & Sweetlove, 2010; Dröse *et al.*, 2011; Bleier & Dröse, 2013). However, in plants, the direct contribution of SDH to mitochondrial ROS production has not yet been established.

In plants, mutations in SDH subunits were associated with changes in development and in ROS homeostasis. Heterozygous *SDH1-1/sdh1-1 Arabidopsis thaliana* showed low SDH activity but increased photosynthesis, nitrogen assimilation and stomatal conductance (Fuentes *et al.*, 2011). However, pollen abortion and reduced seed set were also reported when SDH1-1 levels

were decreased by RNA interference (León *et al.*, 2007). In addition, *A. thaliana* possess an *SDH1-2* gene that is significantly expressed only in roots, and homozygous mutations in this gene do not affect growth or development (León *et al.*, 2007).

In *Solanum lycopersicum*, RNA interference against SDH2-2 decreased SDH activity and increased the rate of photosynthesis, stomatal opening and plant growth (Araújo *et al.*, 2011). Metabolic alterations in organic acid concentrations caused by decreased SDH activity have been suggested to be responsible for these changes in plant development, but the role of SDH as a source of ROS production in plant mitochondria has yet to be considered. In another study, a point mutation in the substrate-binding site of SDH1-1 (mutant *disrupted stress response 1* (*dsr1*)) reduced SDH activity and decreased mitochondrial ROS production in *A. thaliana* (Gleason *et al.*, 2011), but direct ROS formation at the level of the SDH-1 subunits was not tested. In addition, this mutation impairs the salicylic acid (SA)-induced expression of stress-related genes, indicating an important role of SDH-derived ROS in regulating the expression of plant defense genes.

In view of the central role of SDH in mitochondrial metabolism and the importance of ROS in signaling processes, the aim of this study was to evaluate the capacity of SDH to generate ROS in plant mitochondria and to regulate plant development and stress-related gene expression. Our results demonstrated that SDH is a direct source of ROS in isolated mitochondria and that the ROS production rate is proportional to the reduced state of the flavin group of the SDH1 subunit, consistent with the hypothesis that SDH has a similarly direct role in mitochondrial ROS generation in plants as in mammals (Quinlan *et al.*, 2012). The induction of ROS production by noncompetitive SDH inhibitors impaired plant growth without inhibiting mitochondrial oxygen consumption, indicating that this effect is independent of SDH activity and could instead depend on ROS signaling. In addition, this effect was accompanied by the down-regulation of cell cycle genes and the up-regulation of stress-related genes. However, partial SDH inhibition using the competitive inhibitor malonate (MA) decreased hydrogen peroxide (H_2O_2) production and was associated with increased growth of shoots and roots, while impairing the induction of stress-related genes. Our results indicate that SDH is a source of ROS production in plants and that SDH modulates different cellular signaling processes, as well as cell cycle and stress responses, that are essential to normal plant development.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. seeds were germinated in half-strength Murashige & Skoog (MS) medium at 14°C with a 16-h photoperiod. Two weeks after being sown, the *A. thaliana* seedlings were transferred to hydropony in 200-ml plastic cups (three seedlings per cup) filled with 10% MS medium solution. Rice (*Oryza sativa* L. Nipponbare) seeds were germinated in MS medium (Sigma-Aldrich) at 25°C with a 12-h photoperiod. One week after being sown, the rice seedlings were transferred to hydropony in 200-ml plastic cups (three seedlings per cup) filled with Hoagland–Arnon's nutritive solution (Hoagland & Arnon, 1950).

Isolation of mitochondria using a self-generated Percoll gradient

Mitochondria were isolated from the roots of 2-wk-old plants as previously described by Neuburger *et al.* (1982), with some modifications, such as extraction buffer containing 10 mM HEPES/Tris, pH 7.4, 0.3 M mannitol, 2 mM EGTA, 5 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride, 20 mM β-mercaptoethanol, and 0.1% (w/v) fatty acid-free bovine serum albumin (fat-free BSA). The final protein concentration varied from 10 to 20 mg ml^{-1}.

Isolation of protoplasts

Protoplast isolation was performed as described by Chen *et al.* (2006). Protoplast transformation was performed as described by Tao *et al.* (2002). After transformation, protoplasts were incubated for 24–48 h in the dark at 28°C before imaging. Fluorescence was monitored on an Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Japan) equipped with a set of filters capable of distinguishing between green and yellow fluorescent protein (GFP and YFP, respectively) and plastid autofluorescence. The images were captured with a high-sensitivity photomulitiplier tube detector.

$FADH_2/FAD^+$ status measurement

The $FADH_2/FAD^+$ redox state was determined based on autofluorescence using a fluorimeter with (excitation/emission wavelength) Ex/Em = 490 ± 10 nm/530 ± 10 nm (Kunz & Gellerich, 1993). The assay was performed using coupled isolated mitochondria (0.5 mg ml^{-1}) in respiration medium (see the 'Oxygen consumption measurement' subsection). The additions are indicated in the figure legends.

Measurement of SDH activity

The activity of SDH was determined spectrophotometrically using 2,6-dichlorophenol-indophenol (DCPIP) as an artificial electron acceptor and succinate as the substrate (Robinson & Lemire, 1995). The assay was performed at room temperature (25°C) in 1.0 ml of reaction medium containing 20 mM phosphate buffer, pH 7.2, 0.1% Triton X-100, 4 mM sodium azide, and 50 μM DCPIP. Experiments using coupled isolated mitochondria were carried out in 1.0 ml of respiration medium (see the 'Oxygen consumption measurement' subsection) supplemented with 4 mM sodium azide and 50 μM DCPIP.

Blanks were obtained in the absence of succinate. The reaction was started by adding 10 mM succinate using 0.1 mg ml^{-1} of the final protein concentration. The reduction of DCPIP was monitored for 10 min at 600 nm. SDH activity was calculated using

the molar absorption coefficient of reduced DCPIP ($21.0\ mM^{-1}\ cm^{-1}$).

Oxygen consumption measurements

Oxygen consumption rates were measured polarographically using high-resolution respirometry (Oroboros Oxygraph-O_2K; Oroboros Instruments, Innsbruck, Austria). The electrode was calibrated between 0 and 100% saturation with atmospheric oxygen at 28°C. The isolated mitochondria or rice protoplasts ($0.2\ mg\ ml^{-1}$) were incubated with 2.0 m; of the standard respiration buffer containing 0.3 M mannitol, 10 mM Tris-HCl, pH 7.2, 3 mM $MgSO_4$, 10 mM NaCl, 5 mM KH_2PO_4, 0.3 mM β-NAD^+, and 0.1% (v: v) fat-free BSA.

Determination of mitochondrial H_2O_2 release

H_2O_2 release was measured using the Ampliflu Red (Sigma-Aldrich) oxidation method as previously described (Smith *et al.*, 2004). Briefly, mitochondria or protoplasts ($0.2\ mg$ protein ml^{-1}) were incubated in standard respiration buffer (see the 'Oxygen consumption measurement' subsection) or 10 mM MES, pH 6.5, respectively, supplemented with 10 mM Ampliflu Red and 5 units ml^{-1} horseradish peroxidase. Fluorescence was monitored using a fluorimeter at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H_2O_2.

H_2O_2 release was evaluated in rice or *A. thaliana* tissues using 3,3-diaminobenzidine (DAB) staining. Plant tissue was incubated overnight in DAB staining solution ($1\ mg\ ml^{-1}$ DAB in 10 mM MES, pH 6.5) and photographed by digital photography.

Mitochondrial membrane potential ($\Delta\psi_m$) determination

The $\Delta\psi_m$ was measured using the fluorescence signal of the cationic dye safranine O, which is accumulated and quenched inside energized mitochondria (Akerman & Wikstron, 1976). Isolated mitochondria ($0.2\ mg$ protein ml^{-1}) were incubated in standard respiration buffer (see the 'Oxygen consumption measurement' subsection) supplemented with 15 mM safranine O. Two millimolar carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) was used as a positive control to collapse $\Delta\psi_m$. Fluorescence was detected at an excitation wavelength of 495 nm (slit 5 nm) and an emission wavelength of 586 nm (slit 5 nm). Data were reported in arbitrary fluorescence units. Other additions are indicated in the figure legends.

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen, Waltham, MA, USA). Complementary (c)DNA was obtained using the SuperscriptTMII (Life Technologies, Carlsbad, CA, USA) reverse transcriptase system and a 24-polyTV primer (Invitrogen). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). RT-qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Biosystems, Waltham, MA, USA) using SYBR-green intercalating dye for fluorescence detection. The primer sequences and reference genes are listed in Supporting Information Table S1.

Microarray analysis

Microarray data were obtained from the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo; accession no. GSE22942) (Gleason *et al.*, 2011).

Gene ontology analysis

Gene ontology analyses were performed using the web-based tool and database agriGO (Du *et al.*, 2010).

Functional protein association networks

The functional protein association network was created using the STRING database (Snel *et al.*, 2000) and analyzed using MEDUSA (Hooper & Bork, 2005) and VIACOMPLEX software (Castro *et al.*, 2009).

Protein determination

Protein concentrations were determined as described by Bradford (1976) using BSA as a standard.

Statistical analysis

Data were plotted with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and analyzed by one-way ANOVA and *a posteriori* Tukey's test. *P*-values of 0.05 were considered statistically significant.

Results

The noncompetitive inhibition of SDH increases ROS production in plant mitochondria

Previous studies in mammalian mitochondria demonstrate that SDH is a site of ROS production and that this pathway is induced by inhibiting SDH using noncompetitive inhibitors (Chen *et al.*, 2007; Quinlan *et al.*, 2012). To verify whether noncompetitive inhibition of SDH is also able to induce ROS production in plants, leaf and root tissues from *A. thaliana* were treated with 10 μM thenoyltrifluoroacetone (TTFA), and the H_2O_2 content was evaluated by histological staining. DAB staining showed that TTFA induced H_2O_2 production in both leaf and root tissues (Fig. 1a), and the increase of ROS production in leaves treated with TTFA was confirmed by dichlorofluorescein

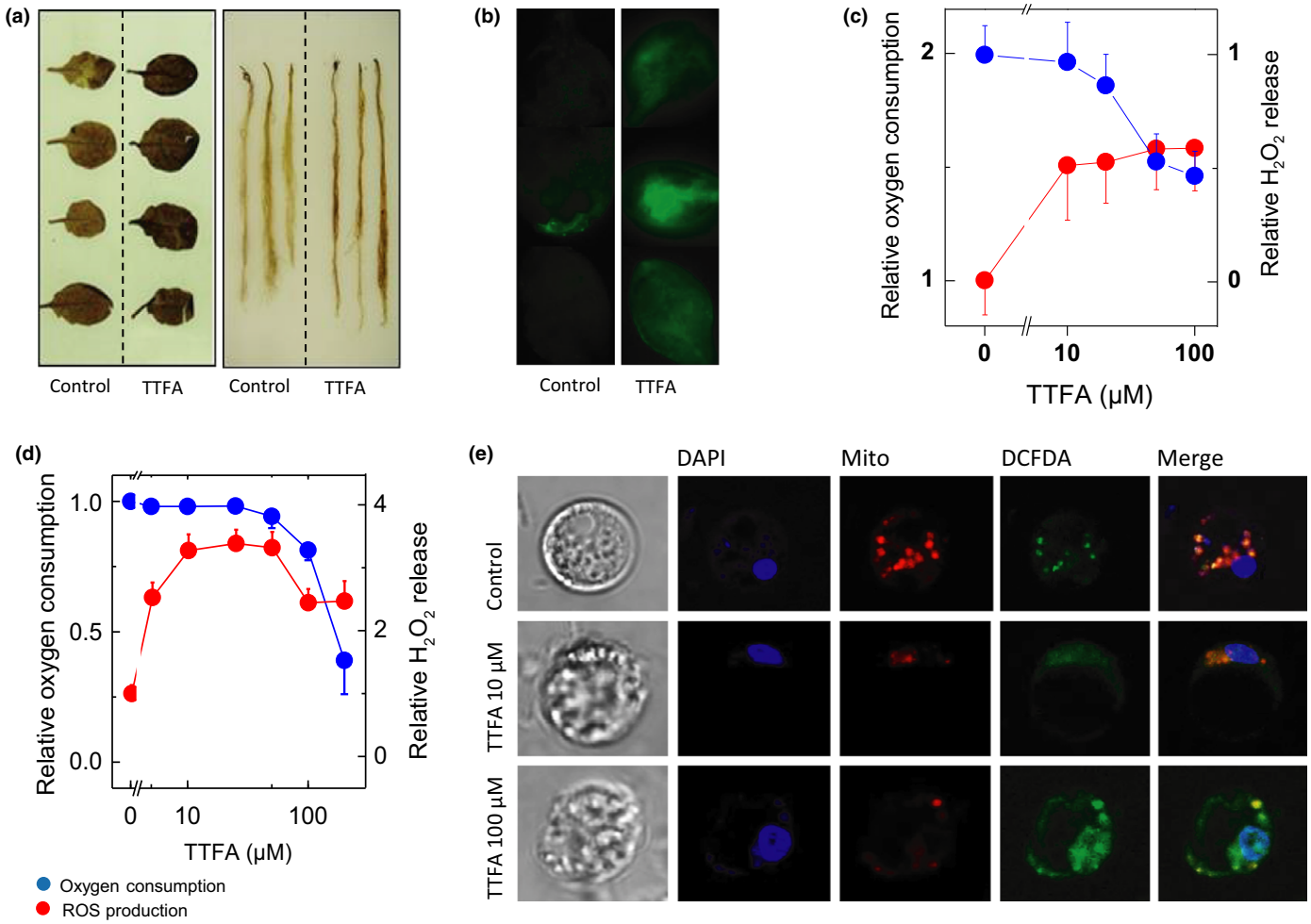

Fig. 1 TTFA induces ROS production in *Arabidopsis thaliana*. (a) 3,3-Diaminobenzidine (DAB) staining indicating reactive oxygen species (ROS) production in tissues treated with 10 μM thenoyltrifluoroacetone (TTFA). (b) Evaluation of succinate dehydrogenase (SDH)-dependent ROS production by dichlorofluorescein diacetate (DCFDA) fluorescence in *A. thaliana* leaves. (c) The effect of increasing concentrations of TTFA on hydrogen peroxide (H_2O_2) release (red) and on oxygen consumption (blue) in *A. thaliana* roots was also quantified fluorometrically. (d) ROS production and SDH-dependent oxygen consumption were also measured in isolated protoplasts in the presence of increasing concentrations of TTFA and confirmed by confocal microscopy of protoplasts treated with 10 and 100 μM TTFA (e). The numeric values represent the mean ± SE of three independent experiments. DAPI, 4′,6-diamidino-2-phenylindole; Mito, mitotracker.

diacetate (DCFDA) staining (Fig. 1b). In addition, the TTFA effect on leaf H_2O_2 production was measured fluorometrically in parallel with oxygen consumption using increasing concentrations of TTFA. Interestingly, a low dose of TTFA was able to induce ROS generation without inhibiting oxygen consumption (Fig. 1c). A similar result was also observed in rice, as shown in the Supporting Information (Fig. S1).

The effect of TTFA on mitochondrial ROS production and oxygen consumption was also confirmed in isolated protoplasts. Noncompetitive SDH inhibition by TTFA was followed by a decrease in succinate-dependent oxygen consumption and an approximately three-fold increase in ROS production (Fig. 1d). As verified in intact leaves, low doses of TTFA were able to increase ROS production without inhibiting oxygen consumption. In addition, confocal microscopy demonstrated that TTFA treatment increased cellular ROS content (Fig. 1e). These results demonstrate that TTFA is able to induce ROS production in plant mitochondria and suggest that, because of its specificity, SDH can be a source of ROS generation, as described in mammalian cells (Quinlan *et al.*, 2012).

To determine the role of SDH in ROS production pathways, the effect of competitive and noncompetitive SDH inhibition on different bioenergetic parameters was evaluated using isolated mitochondria. In the presence of succinate, the substrate of SDH, the competitive inhibitors MA, 3-nitropropionic acid (3NP) and oxaloacetate (OAA) increased the oxidative state of the SDH flavin group, whereas the noncompetitive SDH inhibitors (Q-site inhibitors) TFFA and SA inhibited electron delivery from SDH and increased the reduced state of the flavin group (Fig. 2a). Only the competitive inhibitors blocked succinate oxidation by SDH (Fig. 2b), demonstrating that these inhibitors target the SDH1 subunit. However, all the inhibitors decreased succinate-induced oxygen consumption (Fig. 2c), demonstrating that both types of SDH inhibitors impaired electron transport from succinate to

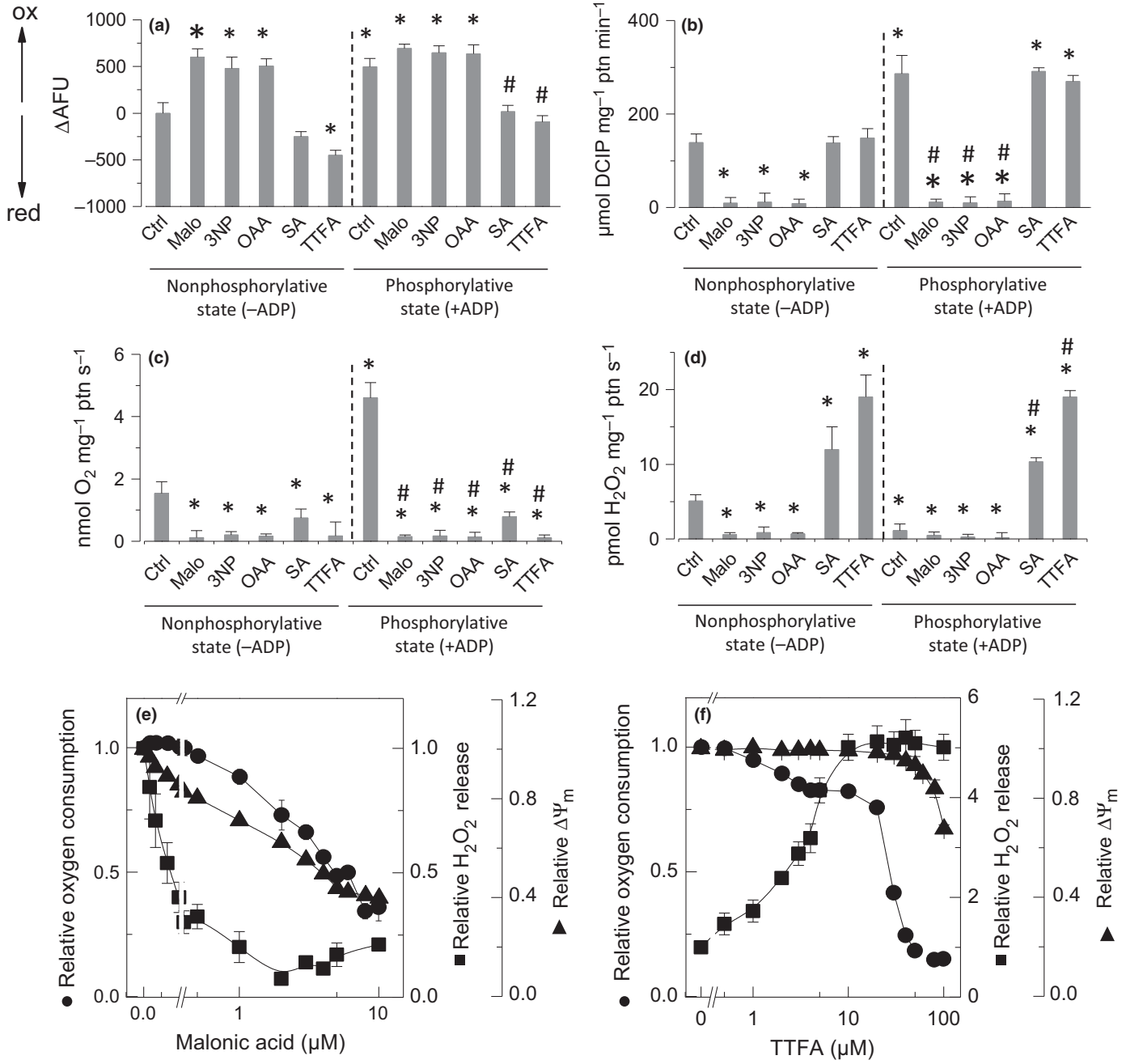

Fig. 2 Effect of competitive and noncompetitive succinate dehydrogenase (SDH) inhibitors on (a) $FADH_2/FAD^+$ redox status, (b) SDH activity, (c) succinate-dependent oxygen consumption, and (d) succinate-dependent reactive oxygen species (ROS) production, in isolated mitochondria. The concentrations of SDH inhibitors were 10 mM malonate (Malo), 1 mM 3-nitropropionic acid (3NP), 1 mM oxaloacetate (OOA), 1 mM salicylic acid (SA) and 100 μM thenoyltrifluoroacetone (TTFA). Succinate-dependent oxygen consumption, ROS production and mitochondrial membrane potential ($\Delta\psi_m$) were evaluated in the presence of increasing concentrations of (e) malonate and (f) TTFA. The reactions were initiated in the presence of 10 mM succinate. The values represent the mean ± SE of eight independent experiments. *, population means are significantly different from the control in the nonphosphorylative state at the 0.05 level; #, population means are significantly different from the control in the phosphorylative state at the 0.05 level. AFU, arbitrary fluorescence unit; Ctrl, control; DCIP, dichlorophenolindophenol; Ox, oxidized; ptn, protein; Red, reduced.

UQ. Nevertheless, the SDH inhibitors produced different effects on succinate-induced ROS production, measured as the release of H_2O_2. The competitive inhibitors, which inhibit succinate oxidation, decreased the ROS production rate, whereas the non-competitive inhibitors increased the ROS production rate (Fig. 2d).

To understand the effect of competitive and noncompetitive SDH inhibition on mitochondrial ROS production, the effect of different concentrations of MA and TTFA, which are specific inhibitors of SDH, on succinate dependent-oxygen consumption, $\Delta\psi_m$ and ROS production was evaluated. Although MA was able to inhibit oxygen consumption as well as membrane

potential and ROS production, the inhibition of oxygen consumption by relatively high concentrations of TTFA was accompanied by a decreased $\Delta\psi_m$ and an increase in the ROS production rate. Interestingly, as demonstrated previously, a low dose of TTFA induced ROS generation without inhibiting oxygen consumption and $\Delta\psi_m$ (Fig. 2e,f).

TTFA induces ROS production by SDH in plant mitochondria

Complex I and complex III are generally considered to be the main sites of mitochondrial ROS production (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010). However, the TTFA-induced ROS production cannot be assigned to complex I and complex III sites. To determine the site of TTFA-induced ROS production, as well as its specificity, different ETS inhibitors and substrates were used. Mitochondrial ROS production was evaluated using succinate, which is specific to SDH activity, as well as pyruvate and malate, which are substrates linked to complex I activity. In addition to MA and TTFA, the effects of rotenone, a complex I inhibitor, and antimycin A and stigmatellin, specific inhibitors of complex III, were also evaluated. Although antimycin A and stigmatellin are both inhibitors of complex III, antimycin A stabilizes the semiquinone radical in the Q-cycle and increases ROS production by complex III, whereas stigmatellin prevents the semiquinone radical step, thereby impairing complex III-dependent ROS production.

As expected, the addition of antimycin A in the presence of pyruvate and malate, substrates that provide NADH for complex I

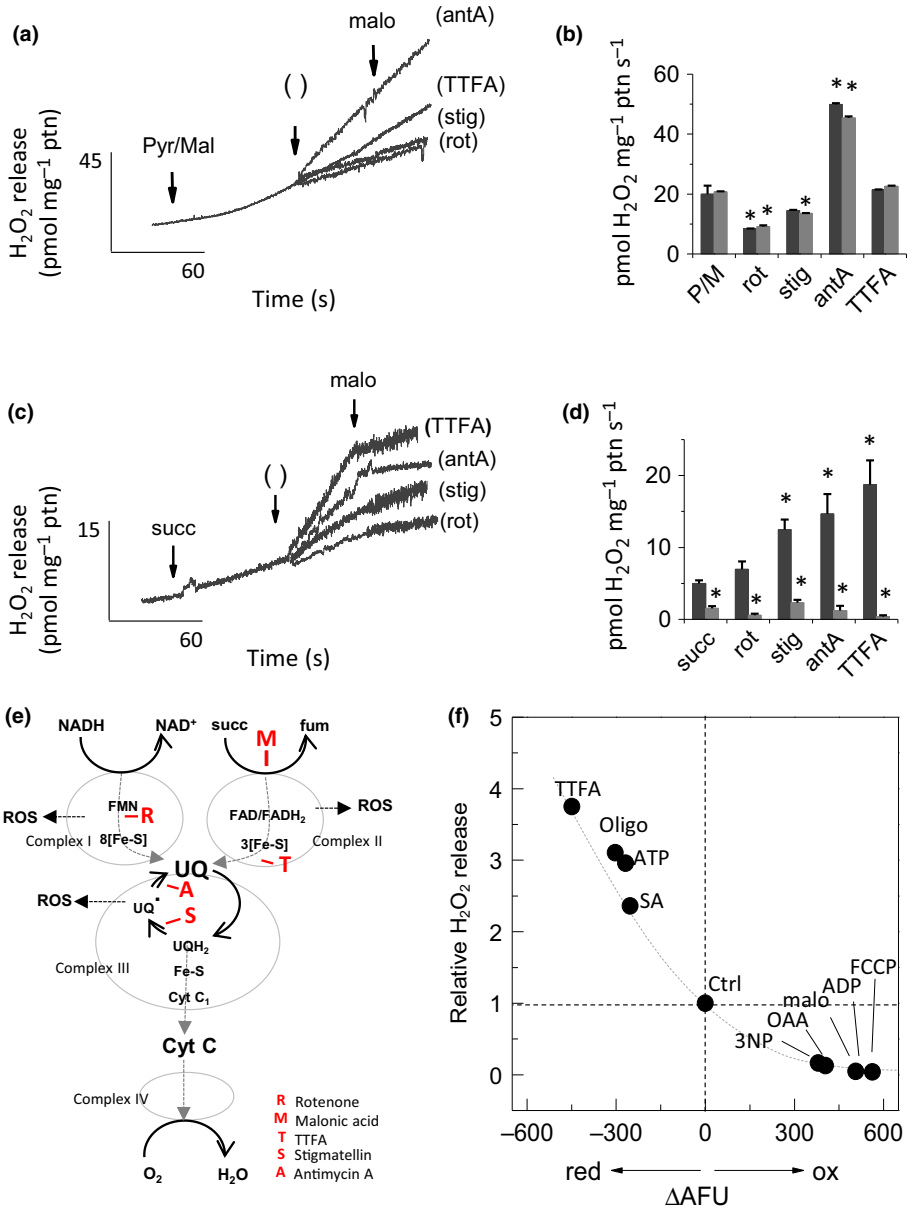

Fig. 3 Mitochondrial oxygen consumption dependent on (a, b) complex I- and (c, d) succinate dehydrogenase (SDH)-linked substrates in *Arabidopsis thaliana* root mitochondria. Dark gray, absence of malonate; light gray, presence of malonate. The reactions were initiated with 10 mM pyruvate/1 mM malate (Pyr/Mal) and succinate (succ). (e) Schematic representation of the electron transport system and the sites of inhibition. (f) The relationship between the SDH redox state and the succinate reactive oxygen species (ROS) production rate in isolated mitochondria. The concentration of inhibitors were 10 mM malonate (malo), 1 mM rotenone (rot), 100 μM thenoyltrifluoroacetone (TTFA), 1 mg ml^{-1} antimycin A (antA), 2 μM stigmatellin (stig), 1 μg ml^{-1} oligomycin (Oligo), 1 mM3-nitropropionic acid (3NP), 1 mM oxaloacetate (OAA) and 1 mM salicylic acid (SA). The carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone (FCCP) and ADP concentrations were 0.5 μM and 1 mM, respectively. The values represent the mean ± SE of eight independent experiments. *, population means are significantly different at the 0.05 level. The empty brackets indicates the point of addition of the inhibitors showed in the right of the graph. AFU, arbitrary fluorescence unit; Cyt C, cytochrome C; FMN, flavin mononucleotide; fum, fumarate; ox, oxidized; Ptn, protein; UQ, ubiquinone; UQH2, ubiquinol.

activity, increased the ROS production rate by *c.* 2.5-fold (Fig. 3a,b). In contrast, the addition of stigmatellin and rotenone decreased the ROS production rate by *c.* 20%. In addition, TTFA or MA did not alter complex I-dependent ROS production. These results confirm that the effects of TTFA and MA on mitochondrial ROS production are specific to SDH activity.

In the presence of succinate as the respiratory substrate, the ROS production rate was also increased *c.* 2.5-fold by antimycin A. However, contrary to what would be expected, the addition of stigmatellin increased the SDH-dependent ROS production rate activity in a similar way to TTFA (Fig. 3c,d). These data demonstrated that TTFA only increases SDH-dependent ROS production, indicating that SDH is a site of ROS production in plant mitochondria. Fig. 3(e) schematically shows the points at which these inhibitors block ETS activity.

The main redox center of SDH is the FAD group, which is bound to the SDH1 subunit, and this prosthetic group is recognized as the site of ROS production in mammalian SDH (Yankovskaya *et al.*, 2003). To determinate the role of the SDH flavin redox state in the ROS production pathways, the relationship between the SDH flavin redox state and SDH-dependent ROS production was analyzed under different conditions. These parameters were evaluated in the presence of competitive SDH inhibitors (3-NP and OAA), noncompetitive SDH inhibitors (SA and TTFA), ATP, which increases the SDH affinity for succinate (Oestreicher *et al.*, 1973), ADP, the substrate of oxidative phosphorylation, oligomycin, an ATP synthase inhibitor, and the H^+ ionophore FCCP, which is able to dissipate the $\Delta\psi_m$. These results demonstrated that the highest rates of ROS production occurred when the SDH flavin was in the reduced state (Fig. 3f).

SDH-dependent ROS production regulates plant growth

To determine the physiological implications of SDH-dependent ROS production, *A. thaliana* germination was evaluated in the presence of TTFA at 10 μM, which induces ROS production without inhibiting SDH activity, and at 100 μM, which both induces ROS production and inhibits SDH activity. Both concentrations were able to impair plant growth (Fig. 4a,b). In addition, the effect of noncompetitive SDH inhibitors on plant growth by hydroponic treatment was also evaluated. In addition to TTFA, which is a specific SDH inhibitor, the effect of nonspecific SDH inhibitors such as SA (Norman *et al.*, 2004) and nitric oxide (NO) (Simonin & Galina, 2013), which perform other biological roles but are also noncompetitive SDH inhibitors, was also examined. Both concentrations of TTFA inhibited shoot and root growth by approximately five-fold, indicating that the inhibitory effect of TTFA on plant growth is not solely dependent on SDH activity inhibition. *S*-Nitroso-*N*-acetylpenicillamine (SNAP), an NO donor, did not inhibit shoot growth, whereas 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO), an NO scavenger, increased shoot growth by 20%. SNAP inhibited root growth by 70%, and this effect was not fully reversed by CPTIO. In addition, SA inhibited shoot and root growth approximately nine- and four-fold, respectively (Fig. 4c,d). Similar effects were also observed in rice plants (Fig. S2).

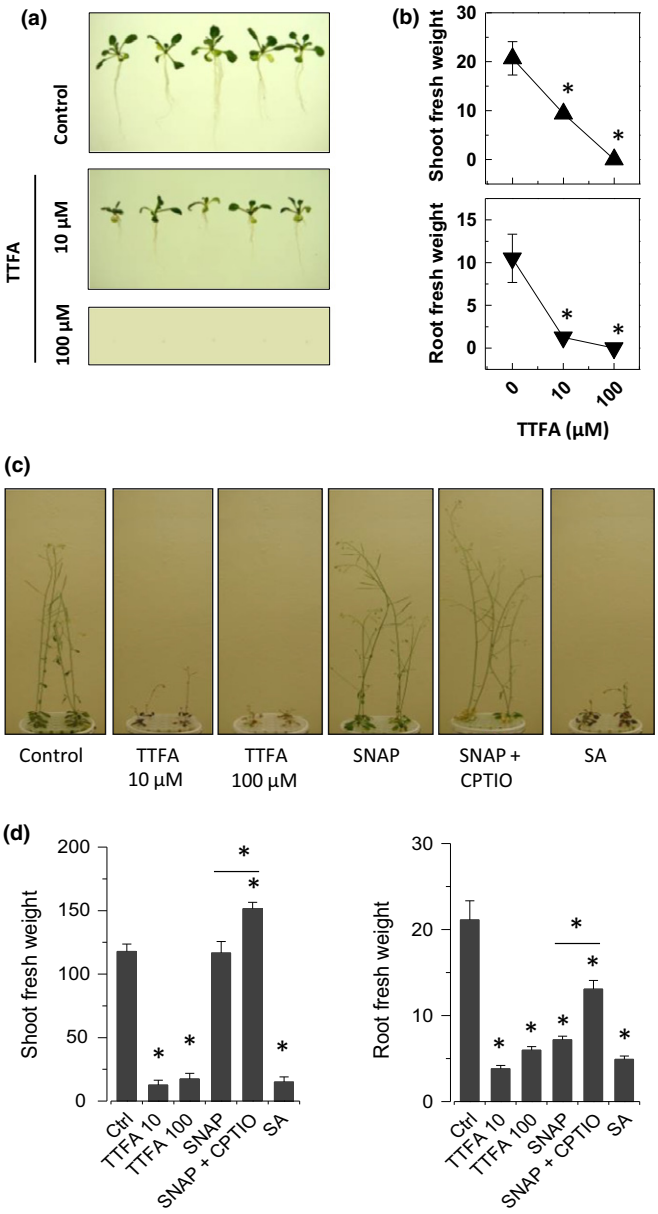

Fig. 4 (a, b) Effect of thenoyltrifluoroacetone (TTFA) on *Arabidopsis thaliana* germination demonstrated in 5-d-old plants. (c, d) In addition, the effect of TTFA and other noncompetitive succinate dehydrogenase (SDH) inhibitors on *A. thaliana* growth was evaluated in 1-month-old plants. The concentrations of the SDH inhibitors were 10 and 100 μM TTFA, 100 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP) and 1 mM salicylic acid (SA). The 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) concentration was 200 μM. The values represent the mean ± SE of three independent experiments with 10 individuals. *, population means are significantly different at the 0.05 level. Ctrl, control.

To determine whether the decreased plant growth was attributable only to the inhibition of electron flow through the ETS, we also evaluated the effect of competitive SDH inhibition on plant growth. Curiously, low doses of MA, a competitive inhibitor of SDH, increased shoot and root growth (Fig. 5a,b), and this effect was accompanied by decreased H_2O_2 release in roots (Fig. 5c). However, high concentrations of MA inhibited oxygen consumption in roots, resulting in reduced plant growth, along

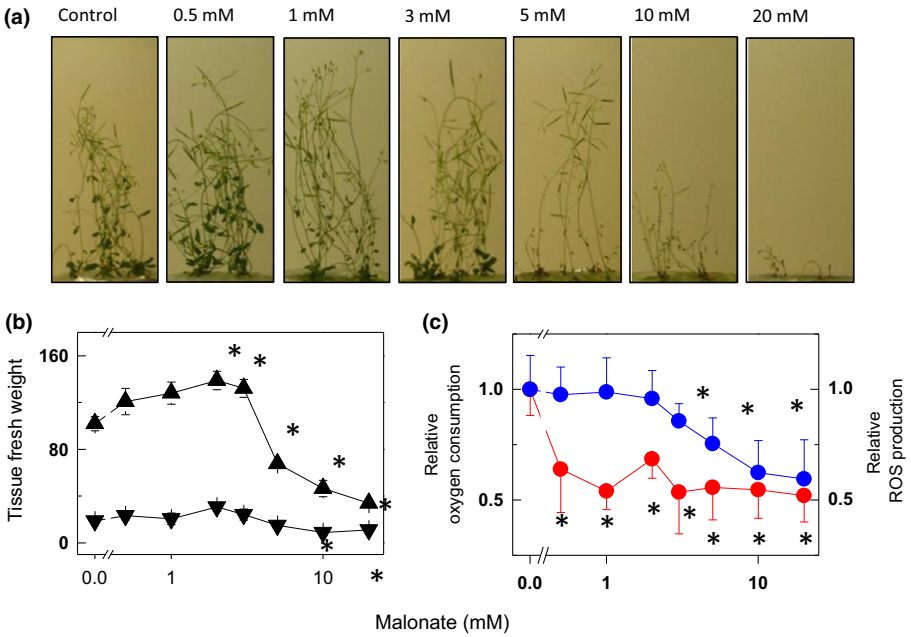

Fig. 5 (a, b) Effect of increasing malonate concentrations on *Arabidopsis thaliana* growth. (c) Effect of malonate on hydrogen peroxide (H_2O_2) release (red) and oxygen consumption (blue) in *A. thaliana* roots. One-month-old plants were used, and the values represent the mean ± SE of three independent experiments with 10 individuals. *, population means are significantly different at the 0.05 level.

with decreased ROS release. A similar result was also previously demonstrated in mutant plants, which showed lower SDH activity accompanied by decreased ROS production and increased growth (Araújo *et al.*, 2011; Fuentes *et al.*, 2011; Gleason *et al.*, 2011).

In rice, low doses of MA were not able to stimulate plant growth or decrease H_2O_2 release, but, as in *A. thaliana*, high MA doses led to reduced oxygen consumption and, consequently, impaired plant growth (Fig. S3). These results demonstrate that the effect of SDH inhibition on plant growth is dependent on the mechanism of SDH inhibition, which modulates mitochondrial ROS production.

ROS production derived from SDH regulates the expression of genes related to plant development and stress responses

To understand the effect of SDH-dependent ROS production on plant growth, the expression of genes related to plant development, including cyclin (*CYC*) genes, cyclin-dependent kinase (*CDK*) genes and histone H4 genes, was verified by RT-qPCR in plants treated with the specific competitive and noncompetitive SDH inhibitors MA and TTFA, respectively. Figure 6(a,b) presents the treatment schemes. For these treatments, 10 μM TTFA was used because, at this concentration, TTFA increases SDH-dependent ROS production without inhibiting SDH activity. TTFA treatment reduced the expression of *AtCYCA3*, *AtCYC3;1*, *AtCDKB2;1* and *AtH4* by approximately three-fold, and this effect was similar to that of direct treatment with H_2O_2 (Fig. 6c). However, pretreatment with MA prevented the inhibitory effect of TTFA on gene expression (Fig. 7d), confirming the requirement for SDH modulation. A similar effect on rice gene expression was also observed (Fig. S4).

Previous studies have demonstrated that the *A. thaliana SDH1-1/sdh1-1* heterozygous and *dsr1* mutants showed reduced SDH activity and, consequently, low mitochondrial ROS production, as well as increased plant growth (Fuentes *et al.*, 2011; Gleason *et al.*, 2011). To verify the effect of SDH activity on the regulation of gene expression, an *in silico* analysis was performed to compare the transcriptional profiles of wild-type (WT) and *dsr1* mutant plants. Among the 22746 genes evaluated, 1798 were up-regulated and 65 were down-regulated in *dsr1* compared with WT (Fig. 6e). Ontological analyses showed that, among the up-regulated genes, those related to biosynthetic processes and nitrogen metabolism were well represented (17% and 14%, respectively). Finally, these results demonstrate that the modulation of SDH activity by mutations or pharmacological inhibitors produces similar effects on the ROS production pathways and, consequently, on the modulation of gene expression.

Gleason *et al.* (2011) demonstrated that *dsr1* mutant *A. thaliana* plants have impaired biotic stress responses. To verify the importance of SDH activity in plant stress responses, the transcription profile of an association network of antioxidant proteins in response to SA was analyzed by comparing WT and *dsr1* plants *in silico*. This network was created using the STRING database (Snel *et al.*, 2000) with genes related to antioxidant metabolism in *A. thaliana*. In WT *A. thaliana*, SA treatment induced the antioxidant response, up-regulating the expression of genes in the association network, compared with plants in the absence of SA. However, in *dsr1* mutant plants, whose SDH activity and ROS concentrations are reduced, this response to SA treatment was impaired (Fig. 7a).

In addition, the relative expression of the genes encoding these antioxidant enzymes was analyzed in response to SA. In WT plants, the up-regulated genes were mainly members of the glutathione-S-transferase (GST) family, but the expression of other genes encoding antioxidant enzymes was also increased in response to stress. In *dsr1* mutants, the antioxidant response is prevented. In addition, the expression of some GST enzymes also decreased (Fig. 7b).

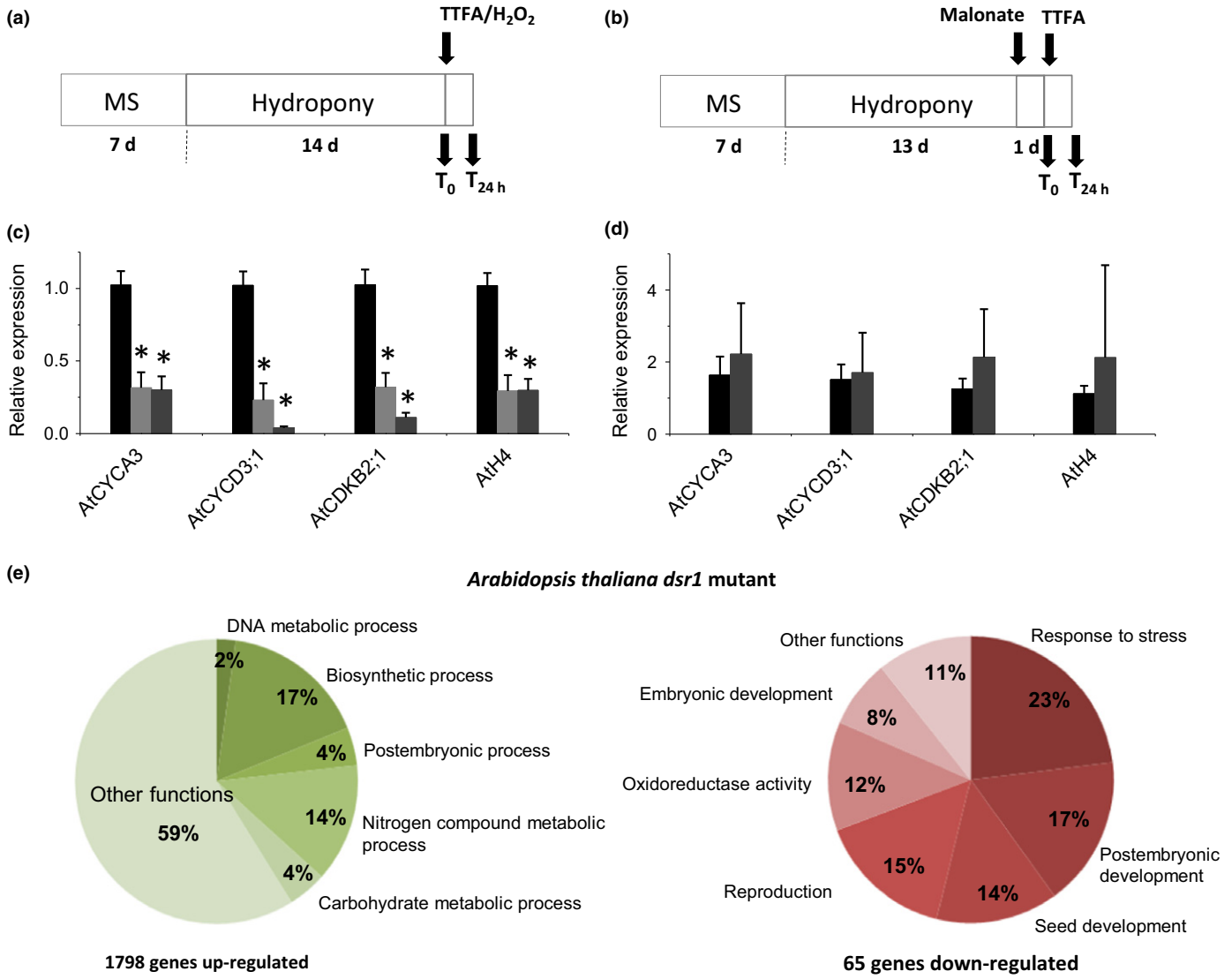

Fig. 6 Quantitative real time-PCR evaluation of the effect of succinate dehydrogenase (SDH) inhibition on developmental gene expression in *Arabidopsis thaliana*. (a, b) Schematic representation of plant treatments. MS, Murashige & Skoog medium. (c) Relative expression of *CYCLIN A3* (*AtCYCA3*), *AtCYCD3;1*, *CYCLIN-DEPENDENT KINASE B2;1* (*AtCDKB2;1*), and histone H4 from *Arabidopsis thaliana* (*AtH4*) in plants treated with hydrogen peroxide (H_2O_2) or 10 μM thenoyltrifluoroacetone (TTFA). Black, control; light gray, H_2O_2; dark gray, TTFA. (d) Relative expression of *AtCYCA3*, *AtCYCD3;1*, *AtCDKB2;1*, and *AtH4* in plants pretreated with 10 mM malonate before TTFA treatment. Black, control; dark gray, TTFA. The values represent the mean ± SE of three independent experiments. *, population means are significantly different at the 0.05 level. (e) The ontological analysis of differentially expressed genes in the *disrupted stress response 1* (*dsr1*) mutant of *A. thaliana*.

To confirm that the modification of antioxidant response-related gene expression observed in mutant plants was attributable to decreased SDH-dependent ROS production and not just to decreased SDH activity, the expression of GST enzymes in response to 10 μM TTFA treatment was experimentally evaluated by RT-qPCR. As with H_2O_2 treatment, TTFA was able to increase *AtGSTF8* expression (Fig. 8a,b). The effect of other non-competitive SDH inhibitors, such as SA and NO, was also evaluated. Both inhibitors induced GST expression, and this effect was impaired by MA pretreatment (Fig. 8c,d), demonstrating the specificity of SDH modulation in this response. In addition, the NO effect was also impaired by CPTIO, an NO scavenger (Fig. 8d). The expression of GST phi classes 5 and 10 (*OsGSTF5* and *OsGSTF10*), which are responsive to oxidative stress (Jain *et al.*, 2010; Gleason *et al.*, 2011), was also evaluated in rice. The effects of competitive and noncompetitive inhibitors on gene expression mimicked those observed in *A. thaliana* (Fig. S5).

Discussion

ETS inhibitors are useful tools for determining different aspects of mitochondrial function, including oxygen consumption, $\Delta\psi_m$ and ROS production. SDH inhibitors can be

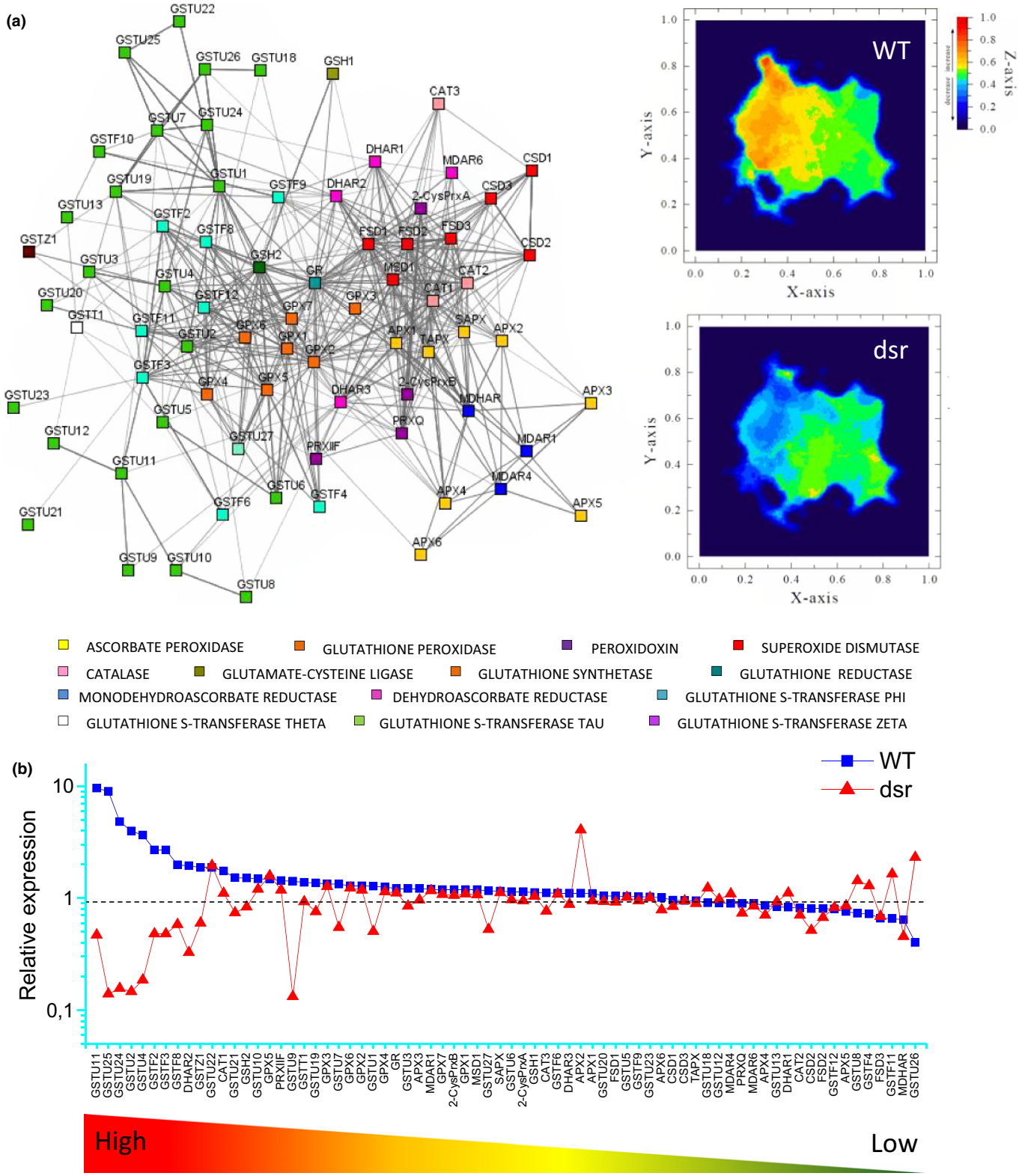

Fig. 7 Effect of salicylic acid treatment on *Arabidopsis thaliana* antioxidant gene network expression. (a) Heatmap demonstrating the antioxidant gene network expression in wild type (WT) and *disrupted stress response 1* (*dsr1*) mutants treated with salicylic acid. (b) Relative expression of antioxidant genes in WT and *dsr1* mutants treated with salicylic acid.

divided into two subgroups: competitive inhibitors that bind to the succinate-binding site, such as MA, 3NP (Alston *et al.*, 1977) and other TCA cycle metabolites, including malate, fumarate, citrate and especially OAA (Gutman *et al.*, 1971; Kearney *et al.*, 1972); and noncompetitive inhibitors that bind to the Q-site, including TTFA (Mowery *et al.*, 1976),

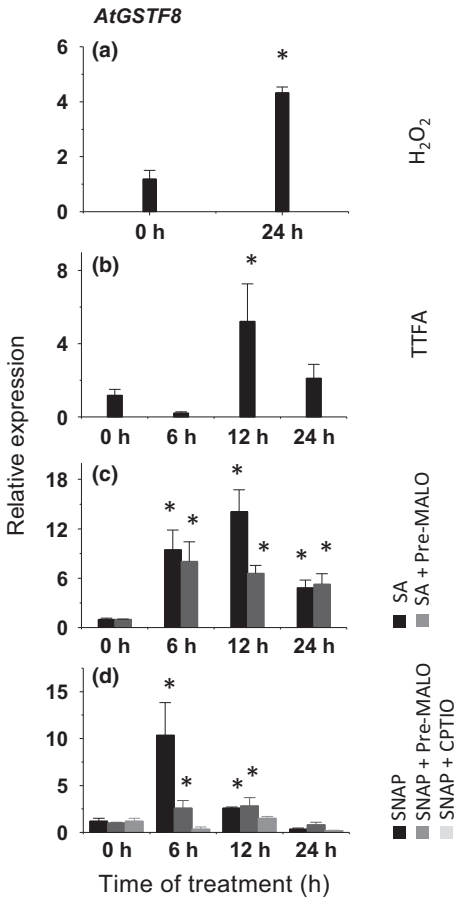

Fig. 8 Evaluation of *GLUTATHIONE-S-TRANSFERASE PHI TYPE 8* (*AtGSTF8*) expression in *Arabidopsis thaliana* treated with (a) hydrogen peroxide (H_2O_2), (b) 10 μM thenoyltrifluoroacetone (TTFA), (c) 1 mM salicylic acid (SA), or (d) 100 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP). The concentrations of malonate (MALO) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (CPTIO) were 10 mM and 200 μM, respectively. The values represent the mean ± SE of three independent experiments. *, population means are significantly different at the 0.05 level.

ATPenins (Miyadera *et al.*, 2003), vitamin E analogs (Dong *et al.*, 2008, 2011a,b), SA (Norman *et al.*, 2004) and NO (Simonin & Galina, 2013).

Previous studies demonstrated that mammalian SDH can be a major source of mitochondrial ROS production when SDH activity is blocked by a noncompetitive inhibitor (Quinlan *et al.*, 2012), and Chen *et al.* (2007) demonstrated that TTFA, a noncompetitive and specific SDH inhibitor, is able to induce ROS production.

In the present study, we have evaluated the capacity of SDH to generate mitochondrial ROS and thus regulate development and stress-related gene expression in plants. Our results demonstrated that SDH is a direct source of ROS in plant mitochondria and that the induction of ROS production by specific SDH inhibitors impairs plant growth. In isolated mitochondria, the competitive SDH inhibitors MA, 3NP and OAA increased the oxidative state of SDH (Fig. 2a) through the inhibition of succinate oxidation (Fig. 2b). This effect was accompanied by decreased mitochondrial H_2O_2 production (Fig. 2c). However, the noncompetitive inhibitors SA and TTFA did not inhibit succinate oxidation (Fig. 2b), increasing the reduced state of SDH (Fig. 2a) and mitochondrial H_2O_2 production (Fig. 2c). In addition, the effect of MA and TTFA on mitochondrial ROS production is specific to SDH modulation and does not depend on other ETS complexes such as complex I and complex III, and the mitochondrial ROS production is proportional to the reduced state of SDH (Fig. 3). These data confirm the central role of SDH in mitochondrial ROS production because, in addition to being essential for the generation of ROS by complex I and complex III (Kowaltowski *et al.*, 2009; Murphy, 2009; Brand, 2010; Møller & Sweetlove, 2010), SDH is a direct source of ROS generation in plant mitochondria. Similar results were previously obtained in mammalian mitochondria, where ATPenins, a noncompetitive inhibitor, increased mitochondrial ROS production (Quinlan *et al.*, 2012), demonstrating that SDH is an important site of ROS production in both animals and plants.

As a consequence of the importance of SDH in mitochondrial energy generation and in ROS production, SDH activity needs to be finely regulated. Endogenous molecules such as SA or NO, which are produced mainly under stress conditions (Raskin, 1992; Delledonne *et al.*, 1998) and perform other signaling roles in plant development and tolerance, are able to regulate SDH activity and induce ROS production (Fig. 2d). Noncompetitive SDH inhibition induced oxidative stress and impaired plant growth (Fig. 4), revealing the influence of SDH-dependent ROS production in plant development. Interestingly, a low TTFA concentration (10 μM) increased mitochondrial ROS production, limiting plant growth without inhibiting SDH activity and, consequently, mitochondrial respiration (Figs 1c,d, 2f). These results demonstrate that the decrease in plant growth was not attributable only to SDH inhibition but was also attributable to the induction of SDH-dependent ROS production by noncompetitive inhibition.

In contrast, competitive SDH inhibition prevented succinate oxidation and, consequently, mitochondrial ROS production (Fig. 2a–d). Thus, a low MA concentration, which did not completely inhibit SDH activity (Fig. 2e), induced plant growth (Fig. 5a). As demonstrated previously in mutant plants (Araújo *et al.*, 2011; Fuentes *et al.*, 2011; Gleason *et al.*, 2011), this effect was accompanied by a decrease in H_2O_2 release from plant tissues without any inhibition of mitochondrial respiration (Fig. 5c).

SDH-dependent ROS production impaired the expression of different genes related to the cell cycle, suggesting a possible mechanism by which ROS induced by SDH/complex II limits plant growth. In *A. thaliana*, TTFA treatment decreased the expression of *AtCYCA3*, *AtCYC3;1*, *AtCDKB2;1* and *AtH4* (Fig. 6c), and this effect was impaired by MA pretreatment (Fig. 6d). A similar result was also observed in rice, in which MA pretreatment induced the expression of CYC and CDK and prevented the TTFA effect (Fig. S4). These data were confirmed by ontological analysis of the transcriptional profiles of WT and the *dsr1* mutant, which shows reduced SDH activity and ROS production, which indicated that the lack of SDH activity induces

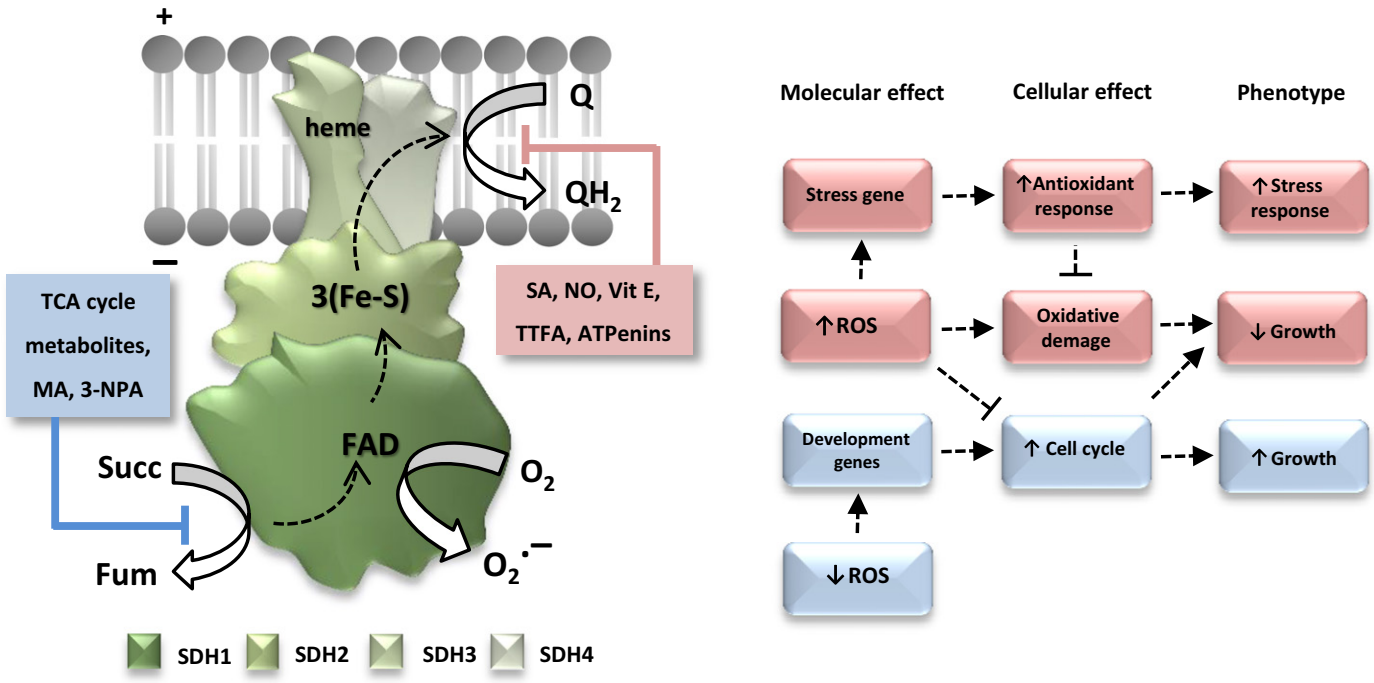

Fig. 9 Effects and phenotypes of succinate dehydrogenase (SDH) inhibition in plants. Noncompetitive inhibitors (pink) such as salicylic acid (SA), nitric oxide (NO), vitamin E, thenoyltrifluoroacetone (TTFA) and ATPenins increase SDH-dependent reactive oxygen species (ROS) production. The increased ROS production leads to oxidative damage and impairs the cell cycle, resulting in decreased growth. However, SDH-dependent ROS production can also signal the antioxidant responses, increasing plant stress responses. In contrast, competitive inhibitors (blue) such as tricarboxylic acid (TCA) cycle metabolites (oxaloacetate, fumaric acid, citric acid and malate), malonate (MA), and 3-nitropropionic acid (3-NP) decrease succinate oxidation and, consequently, SDH-dependent ROS production. This effect decreases plant stress responses but enables the expression of genes related to development, as verified in *disrupted stress response 2* (*dsr1*) mutants. It is noteworthy that full SDH inhibition decreases mitochondrial function, leading to decreased ATP production and the perturbation of organic acids, making unviable gene knockout (KO) plants for SDH. fum, fumarate; Q, ubiquinone; QH2, ubiquinone reduced/ubiquinol; succ, succinate.

the expression of genes specifically related to biosynthetic processes and nitrogen metabolism (Fig. 6e). Finally, these results indicate that SDH can be a limiting factor in plant growth through mitochondrial ROS generation.

Among the genes that were down-regulated in the *dsr1* mutant, a large number were involved in stress responses (Fig. 6e). Furthermore, these plants had an impaired stress response and were more susceptible to fungal and virulent bacterial pathogens (Gleason *et al.*, 2011), indicating the importance of SDH-derived ROS in the expression of plant defense genes. This result was confirmed by analyzing an association network of antioxidant genes in response to SA-induced stress. In WT plants, SA induced the expression of various genes, mainly including GST family members. However, the response to SA was impaired in the *dsr1* mutant (Fig. 7). The importance of SDH-dependent ROS production in stress responses was confirmed by RT-qPCR. The induction of ROS production by noncompetitive inhibitors such as TTFA, SA and NO increased the expression of *AtGSTF8*, and MA pretreatment, which abolished the SDH-dependent ROS production, impaired this effect (Fig. 8), as verified previously in *dsr1* mutants. In rice, the expression of *OsGSTF5* and *AtGSTF10* was also induced by H_2O_2, and noncompetitive SDH inhibitors induced gene expression in an MA-sensitive way (Fig. S5), confirming the importance of SDH activity in this pathway.

In conclusion, SDH is an important site of ROS production in plant mitochondria, in addition to complex I and complex III, which are well known as sites of ROS production. Different molecules can physiologically control SDH-dependent ROS production by modulating SDH activity. Noncompetitive SDH inhibitors, including vitamin E analogs (Dong *et al.*, 2008, 2011a,b), SA (Norman *et al.*, 2004) and NO (Simonin & Galina, 2013), inhibit UQ reduction, thereby increasing the reduced state of SDH and enhancing H_2O_2 release (Fig. 9, pink). This effect impairs the expression of genes related to plant development and limits plant growth. In addition, ROS delivered from SDH can activate the expression of stress-related genes, thereby inducing antioxidant responses and stress tolerance (Fig. 9, pink). Previous studies have demonstrated that SA is a key component in plant resistance, and its production is induced during and following plant stress (McCue *et al.*, 2000; Lewsey *et al.*, 2009). In addition, a deficiency in SA signaling impairs defense responses and increases susceptibility to pathogen attack (Takahashi *et al.*, 2004; Sanchéz *et al.*, 2010; Jovel *et al.*, 2011). NO production is also induced by plant stress, and NO participates in the activation of pathogenesis-related pathways (Delledonne *et al.*, 1998). Thus, we propose that SDH-dependent ROS represents an additional mechanism to explain plant resistance induced by SA or NO. In contrast, competitive SDH inhibitors such as TCA cycle metabolites (Gutman *et al.*, 1971; Kearney *et al.*, 1972), which

are considered biosynthetic intermediates, inhibit succinate oxidation and SDH-dependent ROS production, leading to an increase in the expression of genes related to plant development and growth (Fig. 9, blue). Therefore, we suggest that SDH is a site of ROS generation in plant mitochondria and that SDH plays an important role in regulating plant development and responses to stress.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The noncompetitive inhibition of SDH induces ROS generation in rice tissues.

Fig. S2 The noncompetitive inhibitors of SDH impair germination, and shoot and root growth in rice.

Fig. S3 Low doses of a competitive inhibitor of SDH decrease ROS production in rice tissue and have no effect on plant growth.

Fig. S4 TTFA treatment impairs developmental gene expression in rice.

Fig. S5 The noncompetitive inhibitors of SDH induce *OsGSTF5* and *OsGSTF10* expression in rice.

Table S1 Sequences of primers used in quantitative real-time PCR experiments

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Figure S1

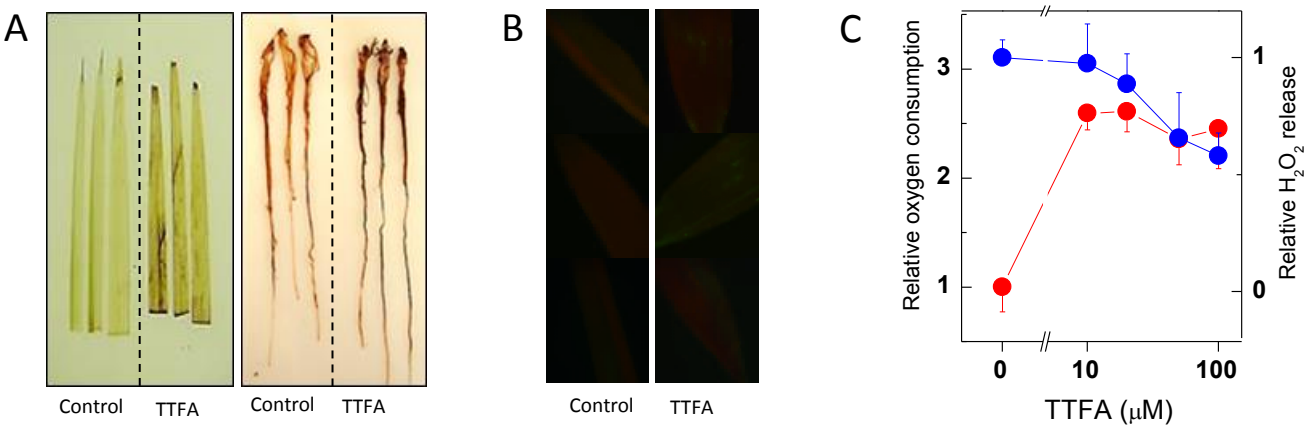

Figure S1: (A) DAB staining indicating ROS production in tissues treated with 10 µM TTFA. (B) Evaluation of SDH-dependent ROS production by DCFDA fluorescence in rice leaves. (C) In addition, the effect of increasing concentrations of TTFA on H_2O_2 release (red) and oxygen consumption (blue) by rice roots was quantified fluorometrically. The numeric values represent the mean ± SE of three independent experiments.

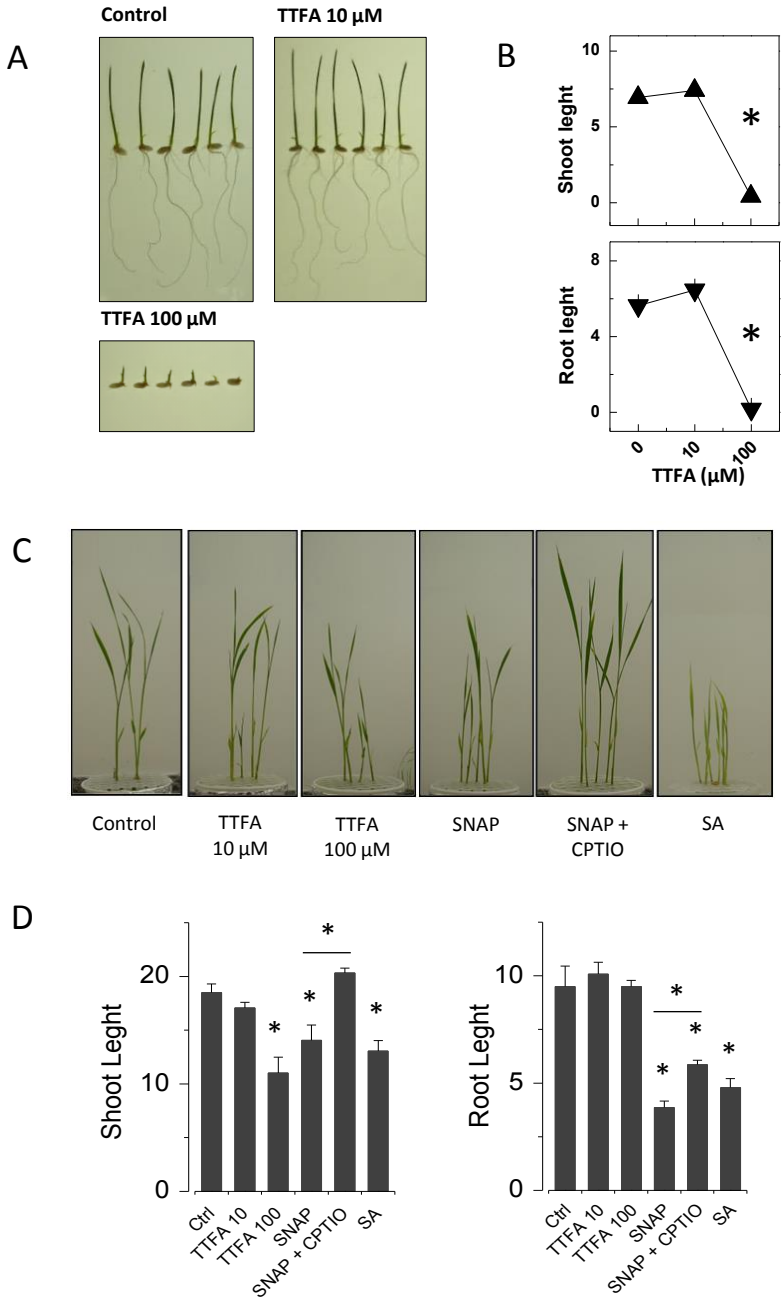

Figure S2: Effect of TTFA on rice germination (A) and (B) in five-day-old plants. In addition, the effect of TTFA and other non-competitive SDH inhibitors on rice growth (C) and (D) was evaluated in one-month-old plants. The concentrations of the SDH inhibitors were 10 and 100 µM TTFA, 100 µM SNAP and 1 mM salicylic acid. The CPTIO concentration was 200 µM. The values represent means ± SE of three independent experiments with ten individuals. *, at the 0.05 level, the population means are significantly different.

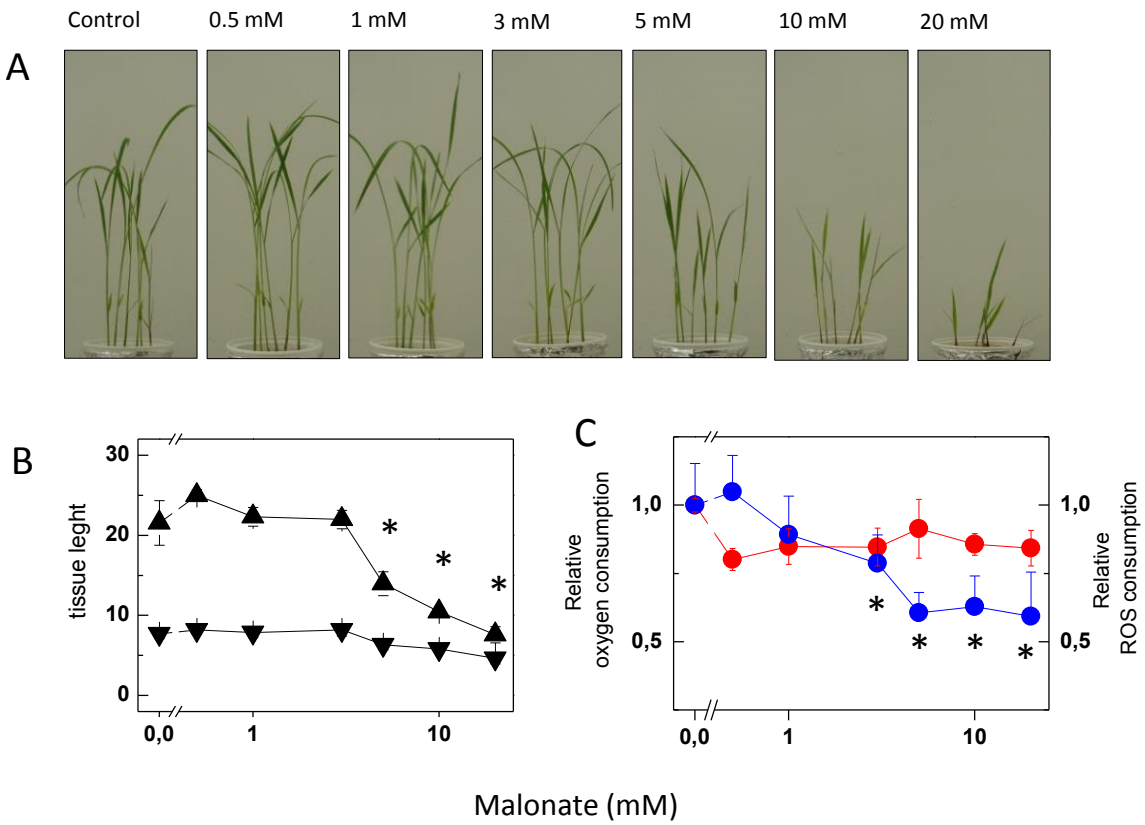

Figure S3: Effect of increasing malonate concentrations on rice growth (A) and (B). (C) Effect of malonate on H_2O_2 release (red) and oxygen consumption (blue) in rice roots. One-month-old plants were used, and the values represent means ± SE of three independent experiments with ten individuals. *, at the 0.05 level, the population means are significantly different.

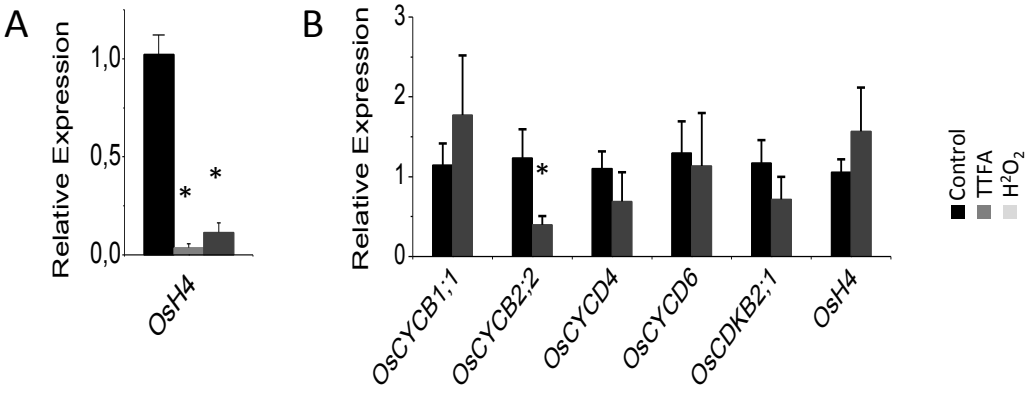

Figure S4: RT-qPCR evaluation of the effect of SDH inhibition on developmental gene expression in rice. (A) Relative expression of *OsH4* in plants treated with H_2O_2 or 10 µM TTFA. Legend: black, control; grey, H_2O_2; dark grey, TTFA. In this condition, the expression of *OsCYCB1;1*, *OsCYCB2;2*, *OsCYCD4*, *OsCYCD4* and *OsCDKB2;1* was not detected. (B) Relative expression of *OsCYCB1;1*, *OsCYCB2;2*, *OsCYCD4*, *OsCYCD4*, *OsCDKB2;1* and *OsH4* in plants pre-treated with 10 mM malonate before TTFA treatment. Legend: black, control; dark grey, TTFA. The values represent means ± SE of three independent experiments. *, at the 0.05 level, the population means are significantly different.

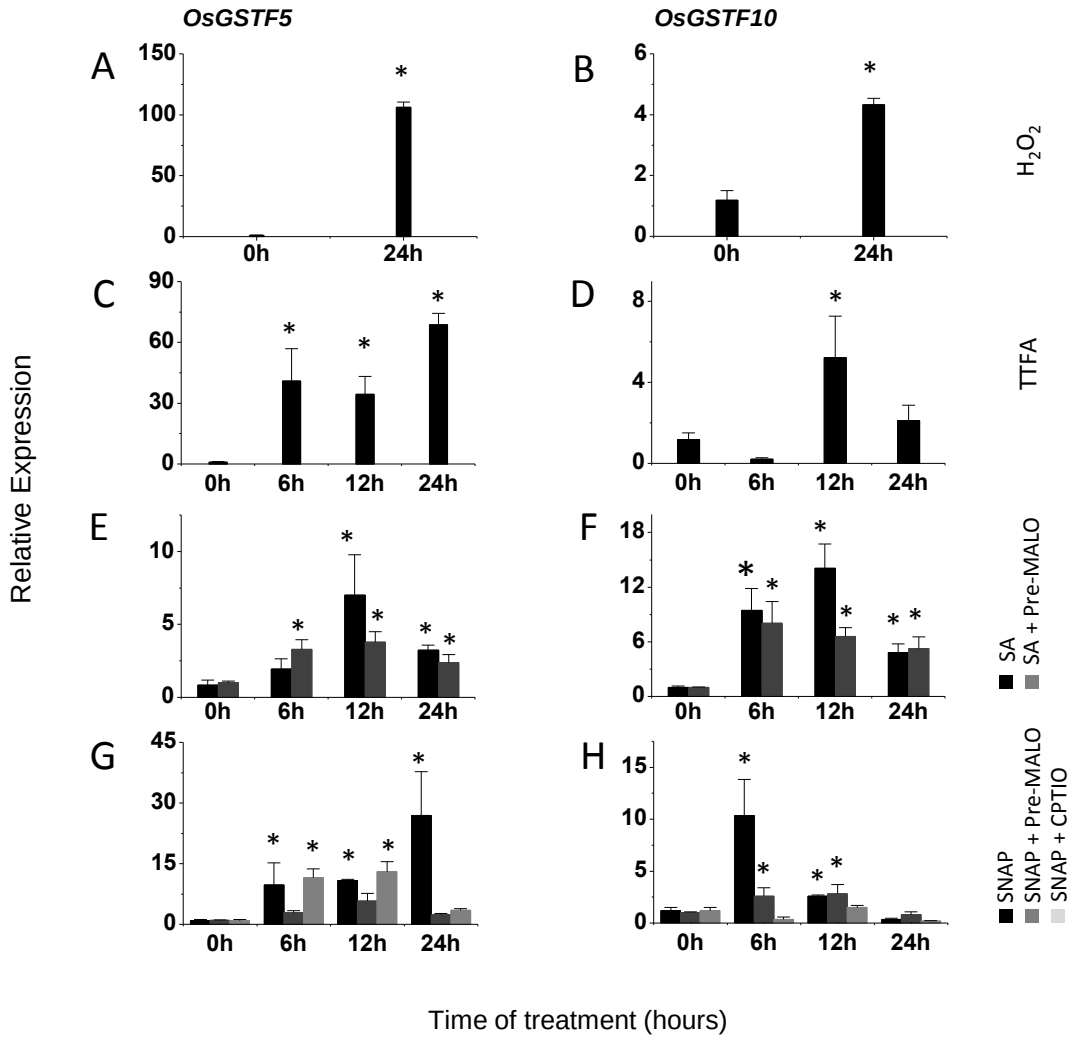

Figure S5: Evaluation of glutathione-S-transferase phi type 5 and 10 (Os*GSTF5* and Os*GSTF10*) expression in rice treated with H_2O_2 (A) and (B), 10 µM TTFA (C) and (D), 1 mM salicylic acid (E) and (F), and 100 µM SNAP (G) and (H). The concentrations of malonate and CPTIO were 10 mM and 200 µM, respectively. The values represent means ± SE of three independent experiments. *, at the 0.05 level, the population means are significantly different.

Table S1: Sequences of primers used in RT-qPCR experiments.

Arabidopsis thaliana		
Cyclins, cyclin dependent kinases and histones		
AtCYCA3	F	CGTCTACTGTGGCTGCTTCA
	R	CGTCTACTGTGGCTGCTTCA
AtCYCD3;1	F	GCAAGTTGATCCCTTTGACC
	R	GCGTTTCTTGGAAGATTGGA
AtCDKB2;1	F	CACACTCAAGAACTGGCATGA
	R	GCTCCATAGCCATCTTTGCT
AtH4	F	GGAGCCAAACGTCATAGGAA
	R	GGCGTCACGGATAACATTCT
Glutathione-S-transferase Phi type		
AtGSTF8	F	GCAAGAAAGTCAAGGCAACC
	R	GAAGGAATCACCAGCCAAGA
Constitutives		
AtEF1α	F	TGAGCACGCTCTTCTTGCTTTCA
	R	GGTGGTGGCATCCATCTTGTTACA
AtUBQ10	F	GGCCTTGTATAATCCCTGATGAATAAG
	R	AAAGAGATAACAGGAACGGAAACATAGT
AtPP2A	F	GTTCTCCACAACCGCTTGGT
	R	TAACGTGGCCAAAATGATGC
Oryza sativa		
Cyclins, cyclin dependent kinases and histones		
OsCYCB1;1	F	TGACAAGGAGATGGAGCACA
	R	TCCAGTGTGGTGCTTGAGAG
OsCYCD2;2	F	CTCAAATTCCAGCCGTCAAT
	R	TAAGCTTCCCATGTCCTGCT
OsCYCD4	F	GCCGTGACTCCATTCTCCTA
	R	GCCTCTGCGATGTCTTCTTC
OsCYCD6	F	AGATGGAGGAAACCGATGTG
	R	CGACGTAGGAGAAGGGAGTG
OsCDKB2;1	F	GCTCGTTCACTGTCCCTCTC
	R	CTGCTGAACCTCGGAATCTC
OsH4	F	GACAACATCCAGGGGATCA
	R	GTGCTCGGTGTAGGTGACG
Glutathione-S-transferase Phi type		
OsGSTF5	F	CCGTTCGGTGAGATTCCAGTA
	R	CGATTGCTCGCGATTGATAG
OsGSTF10	F	ATCTCCCGTAACCCCTTTGG
	R	CAGAGTCAGGTCGCCATCCT
Reference genes		
OsFDH3	F	TTCCAATGCATTCAAAGCTG
	R	CAAAATCAGCTGGTGCTTCTC
OsUBI	F	ACCACTTGCACCGCCACTACT
	R	ACGCCTAAGCCTGCTGGTT
OsACT2	F	GGACGTACAACTGGTATCGTGTT
	R	GTTCAGCAGTGGTAGTGAAGGAG

3.2 CAPÍTULO 2

A EVOLUÇÃO DA SUPERFAMÍLIA DE FUMARATO REDUTASES ENVOLVEU O ESTABELECIMENTO DE DIFERENTES ROTAS DO METABOLISMO PRIMÁRIO E PROCESSOS DE ENDOSSIMBIOSE COM SUBSEQUENTE TRANSFERÊNCIA DE GENES PARA O NÚCLEO

Esse capítulo é referente ao manuscrito “FUMARATE REDUCTASE SUPERFAMILY EVOLUTION ENCOMPASSED THE ESTABLISHMENT OF DIFFERENT METABOLIC PATHWAYS AND ENDOSYMBIOSIS PROCESSES WITH SUBSEQUENT GENE TRANSFERENCE TO THE NUCLEUS”, pronto para a submissão. Neste trabalho, demonstramos que a succinato desidrogenase (SDH), presente em praticamente todos os seres vivos, é membro da família de succinato:quinona oxiredutases, composta também pela enzima fumarato redutase (FRD), presente em bacterias anaeróbicas. Muitos trabalhos vêm demonstrando a importância destas enzimas em diferentes seres vivos. No entanto, a literatura ainda carece de um estudo integrativo desta família, inserida na superfamília de fumarato redutases, composta por outras fumarato redutases, como as fumarato redutases solúveis e dependentes de NADH (FRDS), as fumarato redutases dependente de grupos tiol (TFRD) e as fumarato redutases dependente de L-aspartato, também conhecidas com L-aspartato oxidase (LASPO). Nossos dados demonstram que todas estas enzimas se originaram de um acestral comum, e foram essenciais na evolução das vias metabolicas envolvidas na geração de energia. Além disso, a distribuição destas enzimas nos organismos atuais foi dependente principalmente dos processos de endossimbiose que originaram a mitocôndria e o cloroplasto.

EVOLUTION OF THE FUMARATE REDUCTASE SUPERFAMILY ENCOMPASSED THE ESTABLISHMENT OF DIFFERENT METABOLIC PATHWAYS AND ENDOSYMBIOSIS PROCESSES WITH SUBSEQUENT GENE TRANSFERENCE TO THE NUCLEUS

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ABSTRACT

The organic molecules fumarate and succinate are known to be present in prebiotic systems essential for the origin of life. The reaction pathways involved in fumarate and succinate interconversion have been conserved throughout evolution and are found in virtually all living organisms, including eubacteria, eukaryotes and Archaea. The process of fumarate and succinate interconversion is catalyzed by the enzymes succinate dehydrogenase (SDH) and fumarate reductase (FRD).

SDH, which is found in almost all living organisms, and FRD, which is found in anaerobic bacteria, are members of the succinato:quinona oxireductase family. In spite of their importance for the primary metabolism of different organisms, an integrative study of this family of enzymes is still lacking. They belong to the fumarate reductase superfamily, which is composed of other fumarate reductases such as the soluble fumarate reductase dependent of NADH (FRDS), the soluble fumarate reductase dependent of thiol groups (TFRD) and the L-aspartate oxidase (LASPO), which shows fumarate reductase activity in the absence of oxygen.

Our results demonstrate that these enzymes emerged from a common ancestor and were essential in the evolution of metabolic pathways involved in energy transduction. In addition, the distribution of these enzymes in present day organisms was dependent mainly of endosymbiotic processes, which originated in the mitochondria and chloroplast.

INTRODUCTION

The “Origin of Life” is the natural process which gave rise to living organisms from non-living matter, such as simple organic compounds, and occurred on Earth about 3.5 billion years ago (AWRAMIK, 1992). In this context, a non-enzymatic reverse tricarboxylic acid (TCA) cycle that provided a core mechanism to produce carbon compounds from CO_2 and water under prebiotic conditions has been hypothesized (WACHTERSHAUSER, 1993). Thus, the intermediate molecules in the TCA cycle, such as succinate and fumarate, are present in prebiotic systems of organic molecules essential for the origin of life. Corroborating this hypothesis, the reaction pathways involving fumarate and succinate have been conserved throughout evolution, and enzymes catalyzing their interconversion are found in virtually all living organisms, including eubacteria, eukaryotes and Archaea (HEDERSTEDT AND OHNISHI, 1992).

The succinate:quinone oxidoreductases (SQOR) (EC 1.3.5.1) – succinate dehydrogenase (SDH) and fumarate reductase (FRD) – are oligomeric, potentially reversible isoenzymes that catalyze the interconversion of fumarate and succinate under physiological conditions (HIRSCH *et al*., 1963). SDH activity was first detected in frog muscle in 1909 (THUNBERG, 1909). SDH catalyzes the oxidation of succinate to fumarate (reaction 1), a vital process in organisms that use the TCA cycle for central carbon metabolism. In addition, this reaction is coupled to the reduction of ubiquinone (UQ) to ubiquinol (QH_2) (reaction 2), thereby donating electrons to the aerobic electron transport chain (ETC). On the other hand, FRD is a key component of anaerobic respiration which catalyzes the reverse reactions. Thus, in the absence of oxygen, fumarate acts as a final electron acceptor for anaerobic respiration (BLAUT *et al*., 1989). The interconversion of succinate and fumarate by SQOR enzymes is reversible, besides *in vivo* the oxidation and reduction reactions preferentially occur according to each enzyme (SDH or FRD). In *Escherichia coli*, for example, the FRD is also able to catalyze succinate oxidation; however, with a lower activity (CECCHINI *et al*., 1986; SUCHETA *et al*., 1993).

$$\text{succinate} \rightarrow \text{fumarate} + 2H^+ + 2e^- \quad \text{(reaction 1)}$$

$$\text{quinone} + 2H^+ + 2e^- \rightarrow \text{quinol} \quad \text{(reaction 2)}$$

SDH preferentially catalyzes the oxidation of succinate to fumarate (E° = + 30 mV) using an electron acceptor with a high reduction potential, such as ubiquinone (UQ) (E° = +100 mV). Finally, in the aerobic ETC, oxygen has the high reduction potential (E° = 820 mV), thus operating as a final electron acceptor. On the other hand, FRD catalyzes the reverse reaction, oxidizing an electron donor and reducing fumarate to succinate. For this, the electron donor used by FRD must possess a lower redox potential than the fumarate/succinate. Thus, FRD cannot use UQH_2 as an electron donor, and the fumarate reduction is coupled to the oxidation of a lower reduction potential quinol, such as menaquinol (MQH_2) (E° = -73 mV) (Fig. 1A).

In Gammaproteobacteria, the succinate oxidation and fumarate reduction are carried out by independent enzymes which act as SDH or FRD. On the other hand, in other bacterial clades and in Archaea, only one enzyme with both activities can be found. Eukaryotes possess only one enzyme that acts as a SDH *in vivo*. Despite the large scale genome sequencing projects which have provided SDH and FRD sequences from various organisms, the classification of these enzymes is not obvious because it is based only on biochemical data, which has not yet been determined for many species. Thus, without prior knowledge of the physiological role of a particular enzyme in the metabolism of a given bacterium, it is not possible to predict whether the enzyme functions as a FRD or a SDH *in vivo*.

In helminths, such as *Ascaris suum* and *Haemonchus contortus*, two forms of SQORs that are specific to different growth stages have been identified (ROOS AND TIELES, 1994). During their life cycle, these helminths are able to change from aerobic to anaerobic metabolism. Thus, in these species there are two different oligomeric isoforms of SQOR which lead to a predominant SDH activity during the larval phase (aerobic), and a predominant SDH activity during adult phase (anaerobic) (AMINO *et al*., 2000). This adaptive mechanism is similar

to that described for prokaryotes, such as Gammaproteobacteria, which have both SDH and FRD enzymes. Previous work demonstrated that the Gammaproteobacteria *Escherichia coli* utilizes transcriptional regulators that, in the absence of oxygen, induce the expression of FRD and repress SDH, while in the presence of oxygen, they stimulate the transcription of SDH while repressing FRD expression (PARK *et al*., 1995). As expected, the shift between SDH and FRD expression is also accompanied by changes in the predominant quinone species. In bacteria such as *Escherichia coli,* the combination of SDH and UQ is replaced by FRD and a low-potential quinol, such as MQH_2, during metabolic adaptations to changes in the oxygen supply (COLE *et al*., 1985). On the other hand, helminths use rhodoquinol (RQH_2) (E° = -63 mV) as the low-potential quinol, which is essential to FRD activity (VAN HELLEMOND et al., 1995).

Structurally, SQORs are enzymatic complexes anchored to the cytoplasmic membranes in prokaryotes or the inner mitochondrial membrane in eukaryotes. SDH generally consists of four protein subunits – referred to as SDHA, SDHB, SDHC and SDHD, in bacteria and animals, or SDH1, SDH2, SDH3 and SDH4, in fungi and plants, while FRD consist of FRDA, FRDB, FRDC and FRDD subunits (Fig 1A). The subunit SDHA/FRDA is known as a catalytic flavoprotein, and possesses a bicarboxylate binding site and a covalently bound flavin adenine dinucleotide (FAD). The SDHB/FRDB is classified as an iron–sulfur protein since it contains three iron–sulfur clusters. The subunits SDHC/FRDC and SDHD/FRDD are the hydrophobic subunits which anchor the SQOR complex to the membrane and possess the quinone binding site (YANKOVSKAYA *et al*., 2003).

In many bacteria, the SQORs do not contain the two hydrophobic subunits, but instead contain one larger hydrophobic polypeptide, also named SDHC. Studies of sequence and structure indicate that this larger subunit originated from fusion of the genes encoding the two smaller subunits (HAIGERHALL AND HEDERSTEDT, 1996). In addition, the SQOR membrane bound domain can present zero, one, or two heme prosthetic groups. Thus, the SQOR complexes can be classified into five types, according to the structure of the anchor subunit (LANCASTER, 2001). The anchor domain with two

polypeptides containing two heme groups is denoted as Type A, one polypeptide and two heme groups corresponds to Type B, two polypeptide anchors with one heme group are designated as Type C, whereas anchors with two polypeptides and no heme group are referred to as Type D (HÄGERHÄLL, 1997). Furthermore, there is a “non-classical” SQOR Type E, found in some Archaea such as the Sulfolobales order, which does not contain heme groups. The anchor subunit of the Type E SQOR is non-classical because it is composed of two polypeptide subunits, SDHE and SDHF, which are not found in other types of SQOR complexes (SCHÄFER *et al*., 2002; LEMOS *et al*., 2002) (Fig. 1B).

Previous work demonstrated that the reduction of fumarate is also important in the anaerobic metabolism of eukaryotes such as fungi and some protists. Despite fungi and protists presenting a SDH located in the mitochondria, in these organisms the FRD activity is provided by a monomeric soluble FRD (FRDS) (EC: 1.3.1.6) which possess the conserved catalytic region of SDHA/FRDA (MURATSUBAKI AND ENOMOTO, 1998; COUSTOU *et al*., 2005). This enzyme is also found in the prokaryote *Shewanella* genus (PEALING *et al*., 1992). Curiously, FRDS has no SDH activity *in vitro* and is not able to oxidize quinones. This enzyme is involved in the maintenance of the intracellular redox balance under anaerobic conditions, using NADH as an electron donor for fumarate reduction (BESTEIRO *et al*., 2002).

In methanogenic Archaea, such as Methanobacteria, another soluble fumarate reductase has been found, designated thiol:fumarate reductase (TFRD) (EC: 1.3.4.1). TFRD is composed of two subunits homologous to SDHA/FRDA and SDHB/FRDB, and three additional subunits that are not homologous to SQOR subunits (HEIM *et al*., 1998). The TFRD catalyzes the reduction of fumarate using coenzyme M (CoM-S-H) and coenzyme B (CoB-S-H) as electron donors (BOBIK AND WOLFE, 1989). The reaction catalyzed by the TFRD is indicated below (reaction 3):

fumarate + CoM-S-H + CoB-S-H → succinate + CoM-S-S-CoB (reaction 3)

The heterodisulfide (CoM-S-S-CoB) that is also produced in the last step of the methanogenesis process (BOBIK *et al*., 1987; ELLERMANN *et al*., 1988) is re-reduced by heterodisulfide reductase (HDR) (EC 1.8.98.1) (HEDDERICH *et al*., 1994), in a reaction coupled with ADP phosphorylation (DEPPENMEIER *et al*., 1996). Thus, in Methanobacteria the anabolic reduction of fumarate is indirectly coupled with ATP synthesis. In addition, as demonstrated for FRDS, the TFRDs do not have SDH activity and are unable to oxidize succinate *in vivo* or *in vitro* (HEIM *et al*., 1998).

The increasingly amount of available data related to the structure and function of different FRDs and SDHs in prokaryotes and eukaryotes, and their respective genes, represents an important tool to understand the evolution of these proteins. However, despite the few publications describing independent phylogenetic studies of some FRD/SDH classes, an evolutionary study including the whole family of FRD/SDH proteins is still lacking. Thus, the aim of this study was to determine the phylogenetic relationship among different FRD/SDHs in diverse organisms in order to provide an overview of FRD/SDH evolution and contribute to the understanding of the evolution of this family. Our results demonstrated that FRD/SDH is part of a fumarate reductase superfamily, composed of other fumarate reductases such as NADH:fumarate reductase (soluble fumarate reductase – FRDS), thiol:fumarate reductase (TFRD) and L-aspartate oxidase (LASPO). In addition, the evolutionary history of these proteins encompassed the establishment of different metabolic pathways, endosymbiosis processes and subsequent massive transference of genes from the mitochondrial and chloroplastic genomes to the nucleus.

METHODS

Sequence retrieval

Flavoproteins homologous to SQORs from representative organisms were identified by BLAST (ALTSCHUL *et al*., 1997) searches using databases from the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov), UniProt (www.uniprot.org), Phytozome (www.phytozome.net), Metazome (www.metazome.net) and EuPathDB (www.eupathdb.org). The sequences of SDHA/SDH1 or FRDA from *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Homo sapiens*, *Trypanosoma cruzi*, *Oryza sativa* and *Arabidopsis thaliana* were used as baits.

Sequence alignments and phylogenetic analysis

The protein sequences of the FAD_binding_2 domains (pfam: PF00890), common to all flavoproteins analyzed and identified using SMART tool software (SCHULTZ *et al*., 1998) were used in the phylogenetic analysis. Multiple sequence alignments were performed using multiple sequence comparison by log expectation (MUSCLE) (EDGAR, 2004) as implemented in the molecular evolutionary genetics analysis (MEGA5) software (TAMURA *et al*., 2011) using the default parameters. We visually inspected and edited the alignments to include only unambiguously aligned positions. Phylogenetic analyses were conducted using the Bayesian inference approach as implemented in BEAST 1.8.1 (DRUMMOND *et al*., 2012). The Yule tree was selected as a tree prior to Bayesian analysis, and 50,000,000 generations were performed with Markov chain Monte Carlo (MCMC) algorithms. The best fit of evolutionary models was Dayoff as determined with protTest. The TRACER 1.6 (RAMBAUT *et al*., 2014) was used to check for the convergence of MCMCs and to ensure adequate effective sample sizes (EES > 200) after a burning of 100 generations. We estimated the maximum clade credibility tree with TreeAnnotator, which is part of the BEAST package, and visualized the trees with Figtree 1.3.1

(http://tree.bio.ed.ac.uk/software/figtree/). Statistical support for the clades was determined by assessing the Bayesian posterior probability (PP).

RESULTS AND DISCUSSION

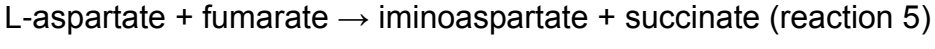
Phylogenetic relationships within the fumarate reductase superfamily

Phylogenetic analyses were carried out with sequences corresponding to the FAD_binding_domains of SQOR, FRDS and TFRD from 180 species representing bacteria, Archaea, plants (Glaoucophyta, Rhodophyta and Viridiplantae), fungi, protists (Excavata and Chromalveolata) and metazoan phyla. Preliminary analysis by BLAST demonstrated that the FAD_binding_domain was also conserved in other proteins, such as L-aspartate oxidase (LASPO) (EC 1.4.3.16), the subunit APRA of adenylyl-sulfate reductase (EC 1.8.99.2) and the subunit GLPB of anaerobic glycerol-3-phosphate dehydrogenase (E.C.1.1.1.8), which are apparently not related to FRD activity (Fig. 2). Thus, these proteins were also included in the phylogenetic analyses, therefore a total of 380 sequences were analyzed. Finally, a total of 354 amino acid residues were included in the final dataset used for phylogenetic tree reconstruction by Bayesian methods. Our results demonstrate that APRA and GLPB appeared as a basal group within the fumarate reductase superfamily (Fig. 3). The GLPB was restricted to Gammaproteobacteria, while APRA was found in both Deltaproteobacteria and Archaea, indicating that APRA was possibly present in an ancestral organism from which both Eubacteria and Archaea originated.

Analyzing the fumarate reductase superfamily, we observed that FRDS appeared as a separate group, subdivided in FRDS from bacteria *Shewanella oneidensis* and FRDS from fungi and protists (Excavata and Chromalveolata). The distribution of FRDS in both prokaryotes and eukaryotes, in addition to the monomeric nature of this enzyme, reinforce the idea that FRDS was present in basal organisms and evolved into other fumarate reductases, such as the multimeric SQOR complex (GEST, 1980). Thus, the FRDS of *Shewanella oneidensis*, fungi and protists might therefore be close related of an ancestral form of FRD.

Interestingly, the phylogenetic analyses indicated that LASPOs, encoded by *nadB* in bacteria and protists, and *fin4* in plants, are included in the fumarate reductase superfamily. LASPOs are able to catalyze the FAD–dependent oxidation of L-aspartate to iminoaspartate, and are the first enzyme in the *de novo* nicotinamide adenine dinucleotide (NAD) biosynthesis by the aspartate oxidase pathway (MORTARINO *et al*., 1996, GRIFFITH *et al*., 1975). LASPOs are required for NAD biosynthesis under both aerobic and anaerobic conditions (MESSNER AND IMLAY, 2002). Under aerobic conditions, LASPOs use oxygen as an electron acceptor, which is then partially reduced to hydrogen peroxide (reaction 4). However, in the absence of oxygen, LASPOs can also act as a L-aspartate:fumarate oxidoreductase, using fumarate as an electron acceptor to produce succinate (MATTEVI *et al*., 1999) (reaction 5). Thus, in the absence of oxygen, LASPOs can have FRD activity (TEDESCHI *et al*., 1996), demonstrating that LASPOs represent another member of the fumarate reductase superfamily. This observation is reinforced by the phylogenetic analysis, which shows that the LASPO and SQOR families form a monophyletic group.

(reaction 4)



(reaction 5)

Our analysis demonstrates that in eukaryotes, LASPOs are found only in photosynthetic clades, with the exception of red algae (Rhodophyta). This fact may suggest that eukaryotes acquired the LASPO during chloroplast endosymbiosis, as proposed by TERNES AND SCHONKNECHT (2014). The chloroplast was originated from an endosymbiotic event during evolution, when a nonphotosynthetic eukaryotic cell took up a photosynthetic cyanobacterium, which evolved into the chloroplast (REYES-PRIETO *et al*., 2007). This endosymbiosis was accompanied by massive gene transfer from the cyanobacterial genome into the nuclear genome of the host cell. It is estimated that in land plants, thousands of genes in the nuclear genome originated from

gene transfer from a cyanobacterial genome (TIMMIS *et al*., 2004). In fact, all plastids found in eukaryotes emerged from a single ancient endosymbiosis event, with few exceptions, such as the *Paulinella chromatophora* plastid, which resulted from a more recent endosymbiosis event (MARIN *et al*., 2005).

Primary endosymbiosis of a cyanobacterium gave rise to the Archaeplastida, including Glaoucophyta, Rhodophyta (red algae) and Viridiplantae (green algae and land plants). In Glaoucophyta and Viridiplantae, the presence of LASPO is well established (KATOH *et al*., 2006), while in Rhodophyta, the aspartate pathway is absence. In addition, LASPO is found in algae protist, such as diatoms (Bacillariophyceae), Oomycota and brown algae (Phaeophyceae). These groups, corresponding to Chromalveolata, acquired their plastid through a secondary endosymbiosis following the uptake of a unicellular red alga (CAVALIER-SMITH, 1999; BAURAIN *et al*., 2010), and this fact raises a question about how Chromalveolata acquired LASPO by endosymbiosis, since the aspartate pathway is absent in Rhodophyta. The answer to this question came from previous works which demonstrated that, in Chromalveolata, thousands of genes potentially originated from green algae, raising a hypothesis that Chromalveolata were subjected to a cryptic secondary endosymbiosis with a green algae before acquiring chloroplasts through Rhodophyta endosymbiosis (MOUSTAFA *et al*., 2009). Thus, the Chromalveolata could have acquired LASPO through gene transference with a green algae.

The phylogenetic analysis indicated that the LASPO from Glaoucophyta and Cyanobacteria is monophyletic, confirming the hypothesis that LASPO was acquired through gene transfer from a cyanobacterial endosymbiont. Curiously, as demonstrated by TERNES AND SCHONKNECHT (2014), the LASPO from Viridiplantae and Chromalveolata, as well as heterokontophyta, are not closely related to cyanobacterial LASPO sequences. The Viridiplantae LASPO is grouped with Bacteroidetes, while Chromalveolata LASPO is grouped with Desulfovibrionales (Deltaproteobacteria) and Halobacteria (Archaea), suggesting that the ancestor of green plants and Chromalveolata replaced the cyanobacterial LASPO through a possible horizontal gene transfer with nonphotosynthetic prokaryotes, as suggested by TERNES AND

SCHONKNECHT (2014). On the other hand, the aspartate pathway is not found in Rhodophyta. Curiously, the phylogenetic analysis of LASPO from Viridiplantae demonstrated that the LASPO from Fabacea species is placed as an outgroup from other Magnoliophyta; however, the reason for this fact has no apparent explanation yet.

Finally, the phylogenetic analysis demonstrated that all SQORs were organized as a single monophyletic group. The first subgroup we identified contained the sequences of subunit A of SQOR type B from Chlamydiae, Firmicutes, Verrucomicrobia, Spirochaetes, Bacteroidetes, Epsilonproteobacteria and Deltaproteobacteria clades. The following groups included sequences relative to TFRD. This topology suggests that TFRD emerged through differentiation of the SQOR complex specifically in methanogenic Archaea such as Methanobacteria. The next group in the phylogenetic tree corresponded to a group that was not very well defined, but was composed of the SQOR type B from Cyanobacteria, the classical Archaeal SQOR type A, the non-classical Archaeal SQOR type E, and the SQOR type D, corresponding to Gammaproteobacteria FRD. Curiously, these analyses indicated that FRD (SQOR type D) from Gammaproteobacteria were closely related to classical SDH from Archaea (SQOR type A). Two isoforms of SQOR, SDH and FRD were found in Gammaproteobacteria, thus these data could indicate that SDH emerged as a duplication of FRD, while the FRD was lost in other species of bacteria. In addition, the FRDA subunit of FRD from the Aquificae bacterial group, which does not possess SQOR activity, was also found in this phylogenetic branch.

In fact, previous work demonstrated that in Aquificae, FRD definitely is not a SQOR. The functional difference than SQOR, is that Aquificae FRD is soluble and uses NADH as an electron donor, working as a member of the enzymatic reductive TCA (rTCA) cycle (MIURA *et al*., 2008). Aquificae are chemolithoautotrophs, which assimilate CO_2 as a unique carbon source through the rTCA cycle (SHIBA *et al*., 1985), which is recognized as an ancestral form of the TCA cycle (AOSHIMA, 2007). The Aquificae FRD consisted of five subunits, designated FRDA, FRDB, FRDC, FRDD and FRDE. Among these, the FRDA and

FRDB subunits presented high sequence identity to the flavoproteins and iron–sulfur subunits of SQOR, respectively (MIURA *et al*., 2008).

Finally, the sequences of flavoproteins of the SQOR type C, which have only SDH activity *in vivo*, appeared as a unique group in the phylogenetic tree. In this group, SDHA from Gammaproteobacteria and Betaproteobacteria appeared grouped in a unique branch, while SDHA from Alphaproteobacteria and from all eukaryotes, seemed to have a common origin. In relation to SDH from eukaryotes, there were groups specific for Excavata, metazoan, Apicomplexa, Heterokontophyta and plants. The Metazoan clade presented a division of SDH from different groups, such as Helminth, Arthropoda, Platyhelminthes/Mollusca and Chordata. Curiously, the $SDHA_A$ from *Ascaris suum*, which is related to FRD activity, appeared as an outgroup of Helminth SDHA, which is related to SDH activity. This data suggests that $SDHA_A$ may have emerged through differentiation of $SDHA_L$, due to the necessity of FRD activity, which would allow the adult worms to live in anaerobic conditions. The same mechanism could also be proposed for subunit SDHD from *Ascaris suum*, which also has two alternative isoforms, $SDHD_A$ and $SDHD_L$.

The SDH1 sequences from Viridiplantae also show interesting features. In the Brassicaceae family there are two isoforms of SDH1 – SDH1-1 and SDH1-2. Our analysis demonstrates that the isoform SDH1-2 forms a separate group of all eudicot sequences, including the isoform SDH1-1. This data suggests that SDH1-2 and SDH1-1 emerged after monocot and eudicot division and before eudicot differentiation, and that SDH1-2 was not maintained in the other eudicot families. Previous work demonstrates that, in *Arabidopsis thaliana*, SDH1-2 is significantly expressed only in the roots, corresponding to less than 10% of SDH1-1 expression. In addition, while SDH1-1 knockout is lethal, the SDH1-2 lack has no effect on the growth and development of homozygous mutant plants, indicating that, in *Arabidopsis*, SDH1-1 is the only functional flavoprotein (LEÓN *et al*., 2007).

Fumarate reductase superfamily gene arrangement in prokaryotes and in the mitochondrial genome

In bacteria and Archaea, the protein components of the SQOR and TFRD complex are typically encoded in a single operon. Thus, the study of gene arrangement, in addition to the previous phylogenetic analysis, can assist in elucidating the evolutionary mechanism of the fumarate reductase superfamily. For this, the arrangement of the operons was analyzed in representative phyla from bacteria and Archaea by consulting the MBGD: Microbial Genome Database (mbgd.genome.ad.jp) (Fig. 4).

In Gammaproteobacteria, SDH and FRD, which correspond to SQOR type C and type D, respectively, are encoded by individual operons. In these species, SDH is encoded by the *SDHC-SDHD-SDHA-SDHB* operon, while FRD is encoded by the *FRDA-FRDB-FRDC-FRDD*. The order C-D-A-B is also found in the SDH from Betaproteobacteria and Alphaproteobacteria, which also corresponds to SQOR type B, reinforcing the phylogenetic results where SQOR from Alpha, Beta and Gammaproteobacteria form a monophyletic group. Curiously, in many species of Alphaproteobacteria, the operon structure is not maintained. While *Rickettsia coxiella* have a typical operon (*SDHC-SDHD-SDHA-SDHB*), in *Rickettsia prowazikii,* the *SDHB* gene is separated from the operon containing the remaining three genes (*SDHC-SDHD-SDHA*). In *Anaplasma marginale*, the SDH operon is divided into two parts, composed of *SDHC-SDHD* and *SDHA-SDHB*. Another exception is *Wolbachia* sp., where the *SDHC* and *SDHD* genes are separated from the operon containing *SDHC-SDHD*. It is important to note that, although there may be some changes in the operon structure in these species, the order C-D-A-B remains conserved in all of them.

The order C-D-A-B is also found in SQORs from Epsilonproteobacteria, Deltaproteobacteria, Spirochaetes, Bacteroidetes, Chlamydiae, Cyanobacterium, Planctomycetes and Firmicutes. In these species, the SQOR corresponds to Type B, thus the gene *SDHD* is absent. However, as

described previously in SQOR Type B, the subunit SDHC corresponds to a fusion between SDHC and SDHD (HAIGERHALL AND HEDERSTEDT, 1996), indicating that the operon involving SQOP type B also follows the C-D-A-B order. In the SQOR type B group there are also species in which the operon structure is not maintained, such as *Rhodothermus marinus* and *Chlamydia trachomatis*, which possess an insertion of an additional gene in the SDH operon, and *Cyanobacterium aponinum,* which do not possess the SDH subunits arranged in an operon. As previously discussed, the classification of SQORs into FRD or SDH is not easy, and in many cases the literature data is contradictory. Due the similarity with SQOR type C operon, and to facilitate the understanding, in this work all genes related to SQOR type B were named as SDH, whether or not they have FRD or SDH activity.

In Archaeal SQOR type A, the operon sequence follows the A-B-C-D order, and was identified in members of phylum Crenarchaeota and Euryarchaeota, such as Thermoproteales, Thermoplasmata, and Archaeoglobi. Unexpectedly, the operon from SQOR type A and SQOR type D, corresponding to Gammaproteobacteria FRD, possessed the same gene arrangement. This fact, in addition to their monophyletic nature, suggests a common origin for SQOR type A and type D. In addition, the presence of this operon in evolutionarily distant organisms indicates that this operon existed before the division between bacteria and Archaea. Thus, we suggest that the operon from SQOR type C results from a duplication of this ancestral operon, which was apparently lost in most bacteria, with the exception of Gammaproteobacteria. Finally, we assume that, as previously described, the fusion of *SDHC* and *SDHD* genes (HAIGERHALL AND HEDERSTEDT, 1996) gave rise to the SQOR type B.

In addition to classical SQORs, there is a non-classical SQOR type E, encoded by the *SDHA-SDHB-SDHE-SDHF* operon, and found only in Sulfolobales. Another division in the fumarate reductase superfamily is the TFRD operon. Its gene arrangement indicates that the TFRD operon from methanogenic Archaea originated from SQOR type E through a deletion of *SDHF* accompanying the *SDHB-SDHC* gene fusion, giving rise to *TFRDB*. One question that remains unanswered: Was the transmembrane domain of the ancestral

SQOR SDHC-SDHD or SDHE-SDHF? In fact, homologs of TFRDB have also been shown in other bacterial and Archaeal enzymes, such as the GLPC subunit of anaerobic glycerol-3-phosphate dehydrogenase (found in Gammaproteobacteria) the subunits HDRB and HDRD from CoB-CoM heterodisulfide reductase (present in methanogenic Archaea and some bacterial species) and in the subunit GLPC from glycolate oxidase (EC 1.1.3.15) (from different bacterial species). The presence of the product of SDHB-SDHF fusion in organisms from different domains indicates that the association between SDHB and SDHF occurred in an ancestral organism, before the bacteria–Archaea division, suggesting the possible presence of the *SDHA-SDHB-SDHE-SDHF* operon in this organism. Thus our results suggest that SQOR type E could be structurally similar to ancestral SQOR, which has been lost in most living organisms, but remained in Sulfolobales.

Interestingly, previous works have demonstrated the presence of SDH genes in the mitochondrial genomes from different eukaryotes (VIEHMANN *et al*., 1996; BURGER *et al*., 1996; GRAY *et al*., 1998; CUENCA *et al*., 2013). Thus, we also evaluated the arrangement of SDH genes in the mitochondrial genome in all eukaryotes analyzed, through data available in NCBI. Our analysis demonstrated the presence of SDH genes in Chromalveolata, Excavata, Rhodophyta and Viridiplantae mitochondrial genomes, but not in fungi or metazoa (Fig. 4). In the Chromalveolata *Rhodomonas salina* we identified the presence of an operon composed of *SDHC-SDHD* in the mitochondrial genome, while *SDHA* and *SDHB* were found in the nuclear genome. In the mitochondrial genome from the Excavata *Reclinomonas americana* we also identified a *SDHC-SDHD-SDHB* operon, while *SDHA* was present in the nuclear genome. The same three subunits of SDH are encoded by the mitochondrial DNA of *Porphyra purpurea*, a photosynthetic red algae; however, without composing an operon. In Rhodophyta, the genes *SDH3* and *SDH2* were also present as an operon in the mitochondrial genomes of *Cyanidium caldarium* and *Chondrus crispus*. On the other hand, in these species, the *SDH1* and *SDH4* genes were nuclear. In Viridiplantae, *SDH3* was found in the mitochondrial genome from *Cycas taitungensis*, *SDH4* was found in the mitochondrial genome from *Marchantia*

polymorpha and *Spirodela polyrhiza*, and both genes were found in the mitochondrial genome from *Cucumis sativus*. In the monocot *Butomus umbellatus*, the four SDH subunits were encoded in the nuclear genome; however, in the mitochondrial genome a pseudogene equivalent to SDH4 subunit was also identified.

The arrangement of SDH genes in the mitochondrial genome demonstrates that these genes, in general, also follow the order C-D-A-B (or 3-4-1-2 in plants). In fact, all SQORs present in eukaryotes are classified as type C. In addition, phylogenetic analysis has previously demonstrated that the SDHA subunits from eukaryotes and Alphaproteobacteria form a monophyletic group. These data reinforce the theory that in eukaryotes, SDH genes were obtained during mitochondria endosymbiosis, and were subsequently transferred to the nuclear genome (BURGER *et al*., 1996). In fact, molecular data support the view that the mitochondria was originated from an endosymbiosis event involving an Alphaproteobacteria–like endosymbiont, being usually assumed that after the endosymbiosis event, there was a massive loss of genes originally encoded in protomitochondrial genome or their transference to nucleus (GRAY, 1992; GRAY, 1993). In this context, most mitochondrial genes were relocated to the nuclear genome, including genes related to components of the ETC, such as SDH (NUGENT AND PALMER, 1991; COVELLO AND GRAY, 1992). Since *SDHA/SDH1* is not present in the mitochondrial genome of any eukaryote, we suggest a very ancient transference of this gene to the nucleus.

Evolutionary model of the SDH/FRD family, and its relationship with species evolution

The evolution of the fumarate reductase superfamily was driven by several instances of differentiation, neo-functionalization, protein association, gene transfer, primary and secondary endosymbiotic gene transfer and horizontal gene transfer from bacteria or Archaea into eukaryotic genomes. The fumarate reductase superfamily evolved from an ancestral flavoprotein, being proposed

that FRDS was present in basal organisms and evolved to actual fumarate reductases, as the quinol–dependent enzymes (BESTEIRO et al., 2002).

It has been recognized that the most basal organisms were heterotrophic anaerobes that obtained energy through fermentation of organic compounds, requiring the constant availability of NAD as an electron acceptor (OPARIN, 1957). The most common form of fermentation is the anaerobic conversion of hexose to lactate (lactic fermentation), however in the primitive anaerobic organisms was also present the “succinic fermentation”, a complex pathway which is still present in both strictest and facultative anaerobes (GEST, 1980). This pathway contains a CO_2 fixation enzyme (PEPCK or pyruvate carboxylase) which converts pyruvate (or phosphoenolpyruvate) to oxaloacetate; a NADH–dependent malate dehydrogenase, which converts oxaloacetate to malate; a fumarase, which converts malate to fumarate; and finally the FRDS, which is able to oxidize NADH and reduce fumarate to succinate. Thus, this pathway results in the reoxidation of two NADH molecules (Fig. 5A). This was an important evolutionary step since it offered the advantage of oxidizing twice as much NADH, requiring 50% less pyruvate to maintain the cellular redox balance, and is also coupled to CO_2 fixation reaction, which later gave rise to photosynthesis and TCA cycle. In this way, the actual FRDS may have the characteristics of an ancestral enzyme, and was conserved in fungi, protists such as Excavata and Heterokontophyta, and in the prokaryote Shewanella genus, preserving the ancestral succinic fermentation in these species.

The next step in SQOR evolution is based on the association of FRD with additional subunits, allowing the binding of FRD to the plasma membrane of prokaryotes, where FRD became part of the anaerobic ETC. In fact, fermentation is quite inefficient as an energy conversion pathway, generating only 2 ATP molecules per molecule of hexose. Thus, the association of FRD with the anaerobic ETC led to an advantageously increased ATP production. It has been supposed that in the transition of FRDS to membrane–bound multimeric FRD (Fig. 5A), additional electron carriers with suitable redox potential were added to the fumarate reduction pathway (THAUER *et al*., 1977). Thus, the reduction of fumarate was associated with the oxidation of MQH2 to menaquinol (MQ), giving

origin to the SQOR family. In the anaerobic ETC, MQ can be re-reduced by a membrane–bound NADH dehydrogenase enzyme, maintaining the link between fumarate reduction and NAD redox balance (Fig. 5C).

Based on our analysis, we suggest that the ancestral SQORs are similar to non-classical SQOR type E from Archaea, encoded by the operon *SDHA-SDHB-SDHE-SDHF*. This operon may have given rise to other enzymes, such as TFRD, that are present in methanogenic Archaea. The TFRD may have originated from deletion of the *SDHF* gene and fusion of the *SDHB* and *SDHE* genes. Fusion of the *SDHB* and *SDHE* genes may further have gave rise the subunits of other enzymes, such as anaerobic glycerol-3-phosphate dehydrogenase, heterodisulfide reductase and glycolate oxidase, which are present not only in Archaea. Posteriorly, may have occurred the replacement of *SDHE* and *SDHF* with *SDHC* and *SDHD*, giving rise to the SQOR type A, and the operon *SDHA-SDHB-SDHE-SDHF* was remained only in Sulfolobaceae family of Archaea. In addition, we propose that the SQOR type A of Archaea and the SQOR type D of Gammaproteobacteria have a monophyletic origin, while the SQOR type C may have arisen as a copy of the original operon, reorganized as *SDHC-SDHD-SDHA-SDHB*, and SQOR type B may have emerged due to fusion between the *SDHC* and *SDHD* genes into a single gene.

Finally, the SDH activity appeared due to the increasing oxygen concentration in the atmosphere, allowing aerobic metabolism. In this context, components of preexisting electron-transport complexes were modified to produce a cytochrome oxidase, which is able to use O_2 as the terminal electron acceptor of the ETC (Fig. 5D). Thus, fumarate could not be used as a final electron acceptor, and, consequently, SQOR acquired SDH activity, oxidizing succinate to fumarate, and contributing to the reduction of quinone to quinol.

Another important step in the evolution of the fumarate reductase superfamily also represents an important step the evolution of life: the emergence of eukaryotic cells and the appearance of mitochondria and chloroplasts. It has been recognized that the mitochondria originated from a primary endosymbiosis of an aerobic Alphaproteobacteria–like endosymbiont by an archaebacterial host,

and subsequently, many mitochondrial genes were transferred to the nuclear genome, including the SDH genes (BURGER *et al.*, 1996) (Fig. 5A). Thus, in many eukaryotic species, the genes for SDH subunits can be found in the mitochondrial genomes. However, one exception is the *SDHA* gene, which was transferred early to the nucleus. In this context, the question related to the fate of the SDH genes from archaebacterial host remains open. Currently, it is not possible to answer this question with complete certainty. However, these genes may have been totally lost by the archaebacterial host before or after the process of endosymbiosis. In addition, only incomplete SDH could be found in Entamoeba and Mastigamoeba. In these organisms, mitochondria had undergone many alterations, generating mitochondrion–related organelles, such as mitosomes. Thus, it is possibly that the SDH gene may have been lost during mitochondrial differentiation.

Another member of the fumarate reductase superfamily is the LASPO, which seems to have emerged through a differentiation of FRDS. In fact, in the absence of oxygen, LASPO behaves as a soluble FRD, but oxidizes L-aspartate instead of NADH. Similar to SQOR, LASPO was obtained by eukaryotes through an endosymbiotic process. LASPO seems to have been acquired later by eukaryotes during chloroplast endosymbiosis between a protozoan host and a Cyanobacteria–like endosymbiont (Fig. 5B). However, no LASPO genes were found in chloroplast genomes, indicating that, similar to *SDHA*, LASPO genes were transferred very early to the nucleus. In addition, FRDS may also have given rise to other proteins, which are not related to FRD activity, such as the subunit APRA of adenylyl-sulfate reductase and the subunit GLPB of anaerobic glycerol-3-phosphate dehydrogenase.

As hypothesized, the molecules succinate and fumarate were present in prebiotic systems, as they were fundamental in ancestral organisms and are indispensable to current life forms (Fig. 6). In many ways, the evolutionary history of the fumarate reductase superfamily is intertwined with the theory of the origin of life and evolution of species. The activities and structures of these enzymes have adapted to different environmental changes, enabling the development of complex metabolic pathways, including fermentation, photosynthesis and

respiration. This plasticity was essential to living organisms, allowing the presence of life in different locations and under a wide variety of environmental conditions.

FIGURES

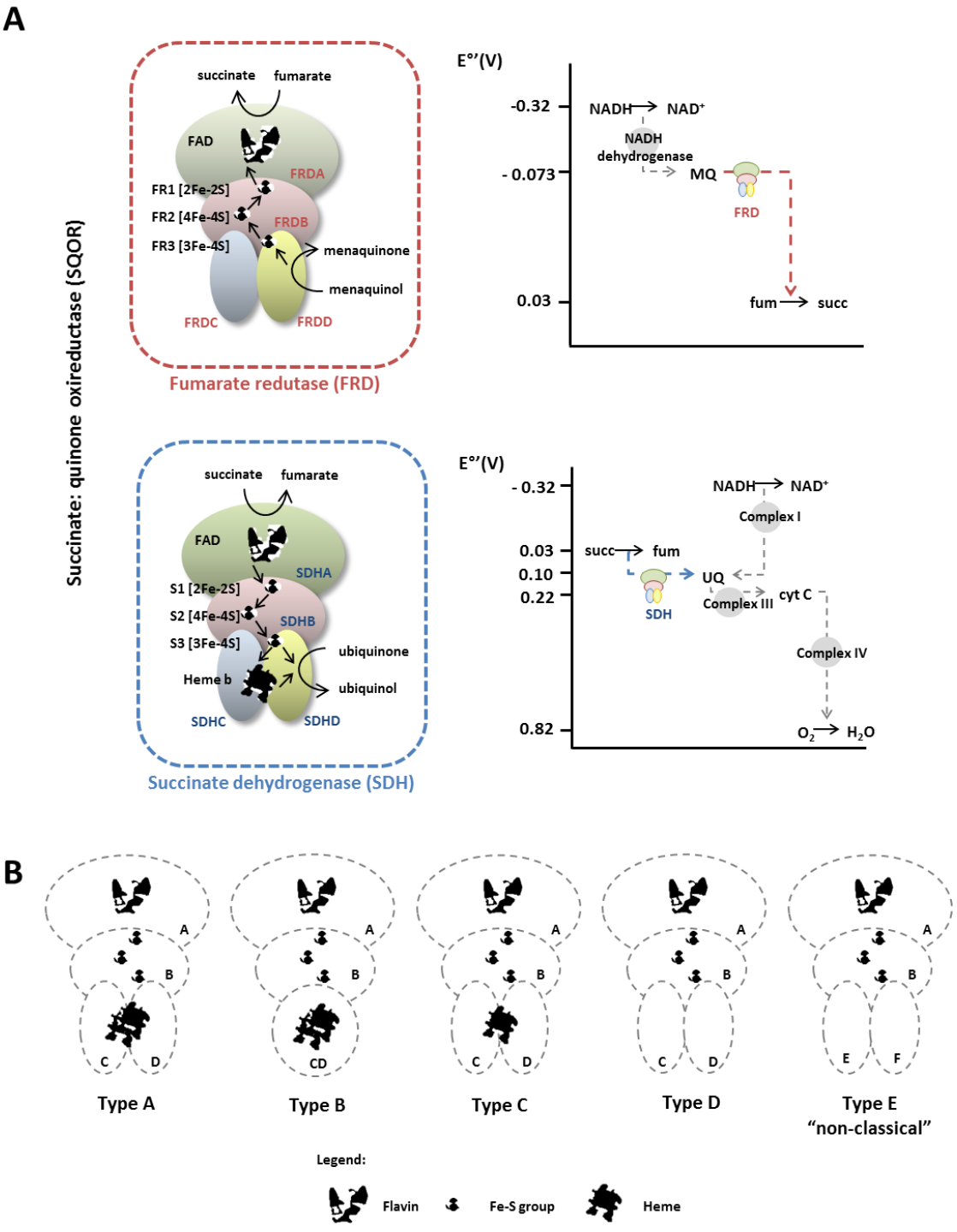

A
Succinate: quinone oxireductase (SQOR)
succinate
fumarate
FAD
FRDA
FR1 [2Fe-2S]
FR2 [4Fe-4S]
FRDB
FR3 [3Fe-4S]
menaquinone
menaquinol
FRDC
FRDD
Fumarate redutase (FRD)
E°'(V)
-0.32
- 0.073
0.03
NADH
NAD+
NADH dehydrogenase
MQ
FRD
fum
succ
succinate
fumarate
FAD
SDHA
S1 [2Fe-2S]
S2 [4Fe-4S]
SDHB
S3 [3Fe-4S]
ubiquinone
ubiquinol
Heme b
SDHC
SDHD
Succinate dehydrogenase (SDH)
E°'(V)
- 0.32
0.03
0.10
0.22
0.82
NADH
NAD+
Complex I
succ
fum
UQ
SDH
Complex III
cyt C
Complex IV
O2
H2O
B
A
B
C
D
CD
E
F
Type A
Type B
Type C
Type D
Type E
"non-classical"
Legend:
Flavin
Fe-S group
Heme

Figure 1. Schematic representation of succinate:quinone oxidoreductase family. *(A) Schematic representation of FRD and SDH activities. In graphics, at the right, are represented the reduction potential of different components of aerobic and anaerobic electron transporter chains. The hydrophilic subunits A and B are schematically drawn in green and red, and the hydrophobic subunits C and D in blue and yellow, respectively (B) Classification (type A-E) of succinate:quinone oxidoreductases (SQOR) based on their hydrophobic domain and heme content. UQ – ubiquinone; cyt C – cytochrome C; MQ – menoquinone.*

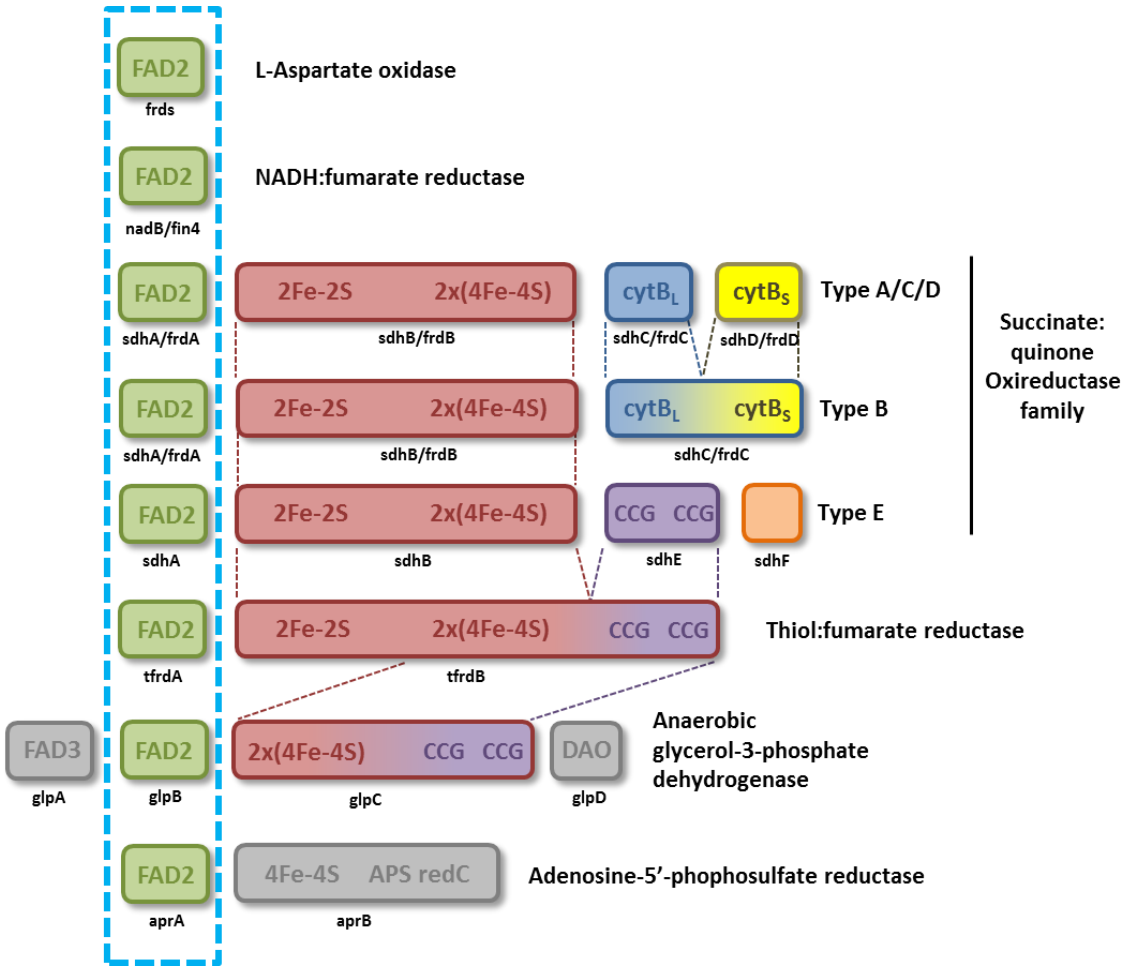


Figure 2. Schematic alignment of FRDS, LASPO, SQOR, TFRD, anaerobic glycerol-3-phosphate dehydrogenase and adenosine-5'-phophosulfate reductase protein domains. *The dotted lines indicate the conserved subunits and the dotted rectangle, indicate the common flavoproteins.*

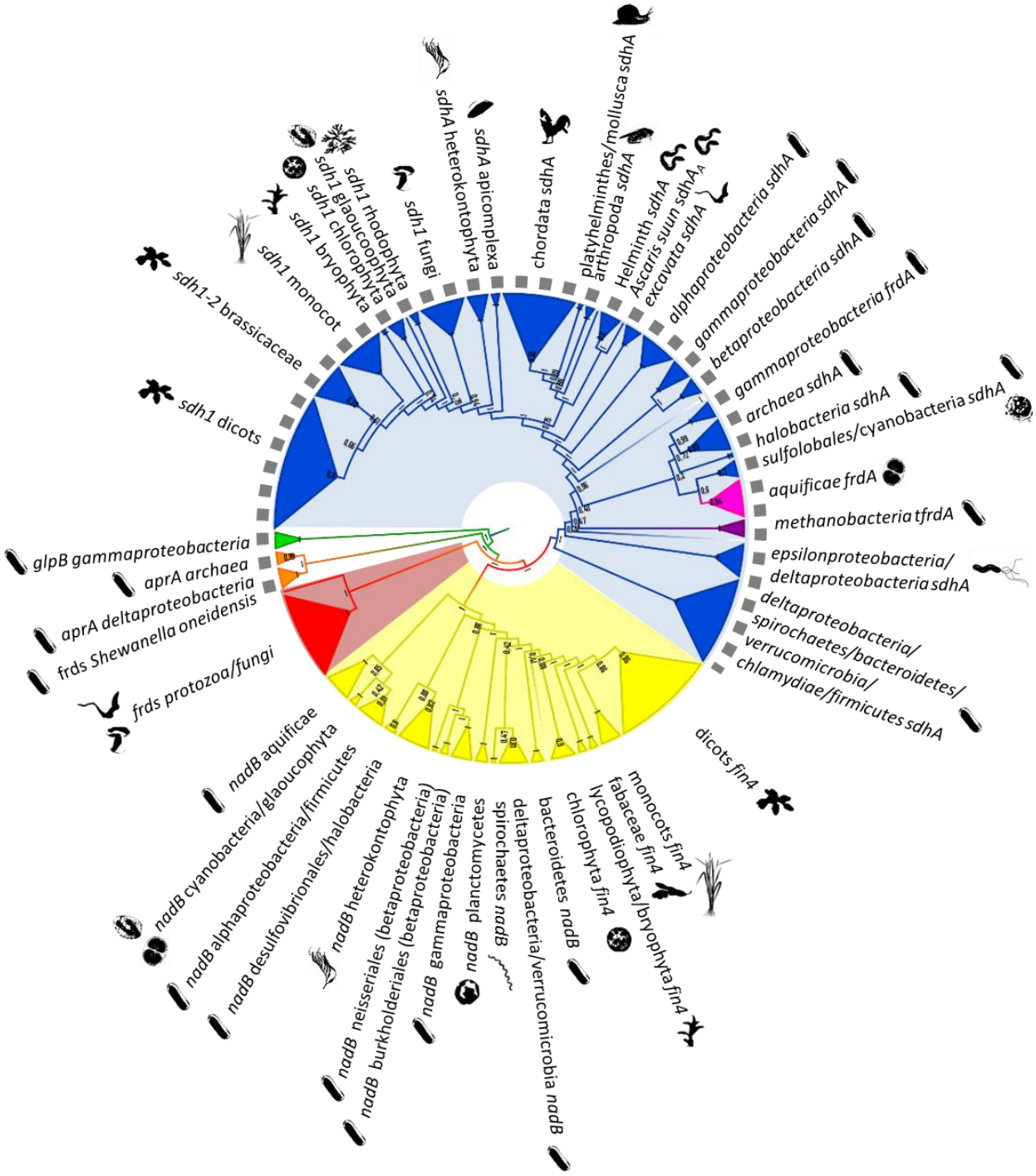

sdh1-2 brassicaceae
sdh1 dicots
sdh1 monocot
sdh1 bryophyta
sdh1 chlorophyta
sdh1 glaucophyta
sdh1 rhodophyta
sdh1 fungi
sdhA heterokontophyta
sdhA apicomplexa
chordata sdhA
platyhelminthes/mollusca sdhA
arthropoda sdhA
Helminth sdhA
Ascaris suun sdhA
excavata sdhA
alphaproteobacteria sdhA
gammaproteobacteria sdhA
betaproteobacteria sdhA
gammaproteobacteria frdA
archaea sdhA
halobacteria sdhA
sulfolobales/cyanobacteria sdhA
aquificae frdA
methanobacteria tfrdA
epsilonproteobacteria/
deltaproteobacteria sdhA
deltaproteobacteria/
spirochaetes/bacteroidetes/
verrucomicrobia/
chlamydiae/firmicutes sdhA
dicots fin4
monocots fin4
fabaceae fin4
lycopodiophyta/bryophyta fin4
chlorophyta fin4
bacteroidetes nadB
deltaproteobacteria/verrucomicrobia nadB
spirochaetes nadB
nadB planctomycetes
nadB gammaproteobacteria
nadB burkholderiales (betaproteobacteria)
nadB neisseriales (betaproteobacteria)
nadB heterokontophyta
nadB desulfovibrionales/halobacteria
nadB alphaproteobacteria/firmicutes
nadB cyanobacteria/glaoucophyta
nadB aquificae
frds protozoa/fungi
frds Shewanella oneidensis
aprA deltaproteobacteria
aprA archaea
glpB gammaproteobacteria

Figure 3. Phylogenetic relationship between fumarate reductase superfamily. ***C****lusters corresponding to three well-supported main family classes were indicated with backgrounds colored in red (FRDS), yellow (LASPO), and blue (SQOR). The activities of proteins belonging to the superfamily are represented by different colors: anaerobic glycerol-3-phosphate dehydrogenase (glpB), in green; adenylyl-sulfate reductase (aprA), in orange; FRDS, in red; LASPO, in yellow; SQOR, in blue; TFRD, in purple and NADH-dependent fumarate reductase from Aquificae, in pink. The dotted grey line indicates the subunits of complex components, while the absence indicates monomeric proteins.*

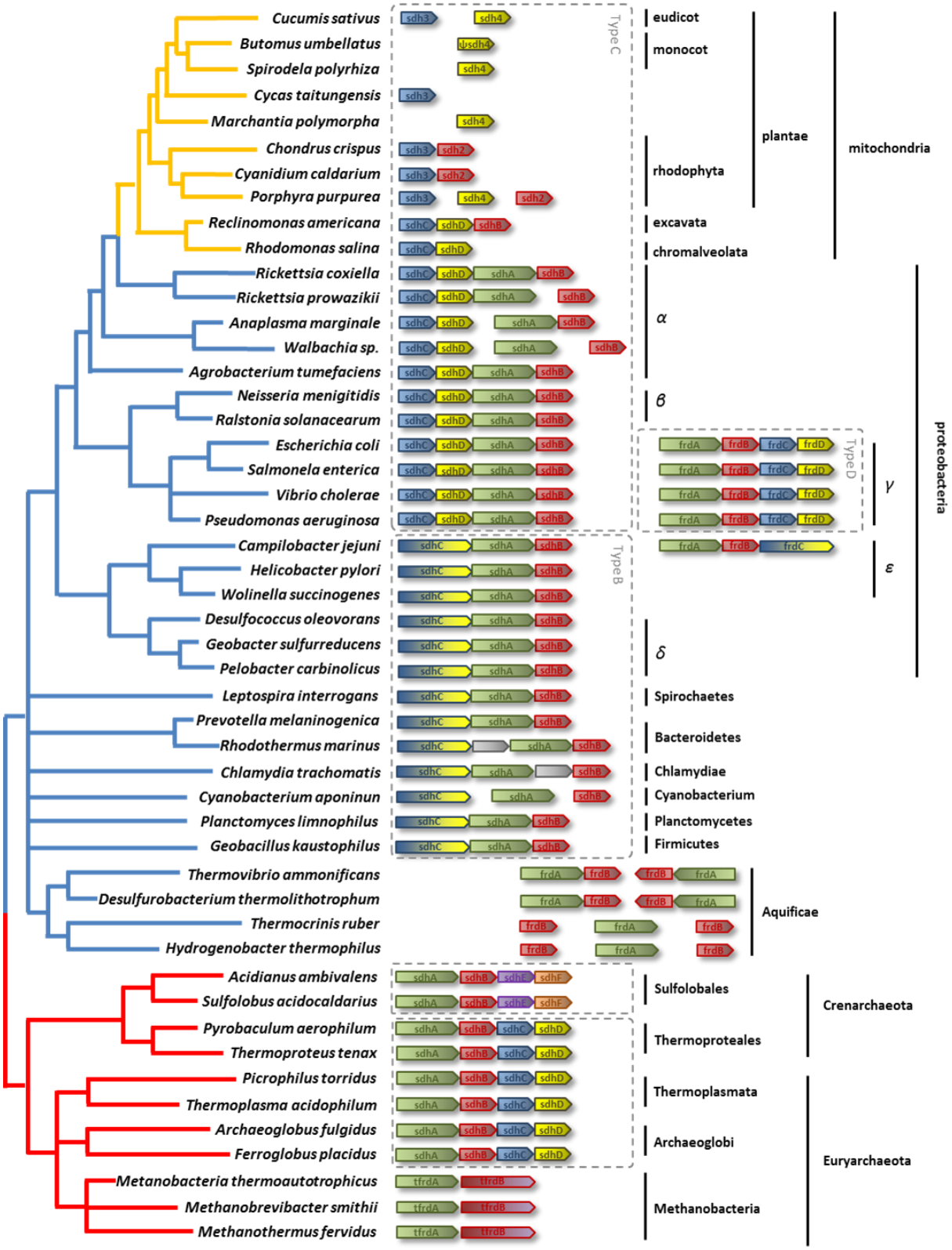

Cucumis sativus
Butomus umbellatus
Spirodela polyrhiza
Cycas taitungensis
Marchantia polymorpha
Chondrus crispus
Cyanidium caldarium
Porphyra purpurea
Reclinomonas americana
Rhodomonas salina
Rickettsia coxiella
Rickettsia prowazikii
Anaplasma marginale
Walbachia sp.
Agrobacterium tumefaciens
Neisseria menigitidis
Ralstonia solanacearum
Escherichia coli
Salmonela enterica
Vibrio cholerae
Pseudomonas aeruginosa
Campilobacter jejuni
Helicobacter pylori
Wolinella succinogenes
Desulfococcus oleovorans
Geobacter sulfurreducens
Pelobacter carbinolicus
Leptospira interrogans
Prevotella melaninogenica
Rhodothermus marinus
Chlamydia trachomatis
Cyanobacterium aponinun
Planctomyces limnophilus
Geobacillus kaustophilus
Thermovibrio ammonificans
Desulfurobacterium thermolithotrophum
Thermocrinis ruber
Hydrogenobacter thermophilus
Acidianus ambivalens
Sulfolobus acidocaldarius
Pyrobaculum aerophilum
Thermoproteus tenax
Picrophilus torridus
Thermoplasma acidophilum
Archaeoglobus fulgidus
Ferroglobus placidus
Metanobacteria thermoautotrophicus
Methanobrevibacter smithii
Methanothermus fervidus
Type C
Type B
Type D
eudicot
monocot
rhodophyta
plantae
mitochondria
excavata
chromalveolata
α
β
γ
ε
δ
proteobacteria
Spirochaetes
Bacteroidetes
Chlamydiae
Cyanobacterium
Planctomycetes
Firmicutes
Aquificae
Sulfolobales
Thermoproteales
Crenarchaeota
Thermoplasmata
Archaeoglobi
Euryarchaeota
Methanobacteria

Figure 4. Arrangement of Sdh/Frd genes in bacterial, archaeal and mitochondrial genomes. *The red lines indicate genes from archaea, the blue lines from bacteria and the orange lines indicated those genes present in mitochondrial genome. The classes A, B, C, D and E of succinate:quinone oxidoreductases are indicate in dotted rectangles,*

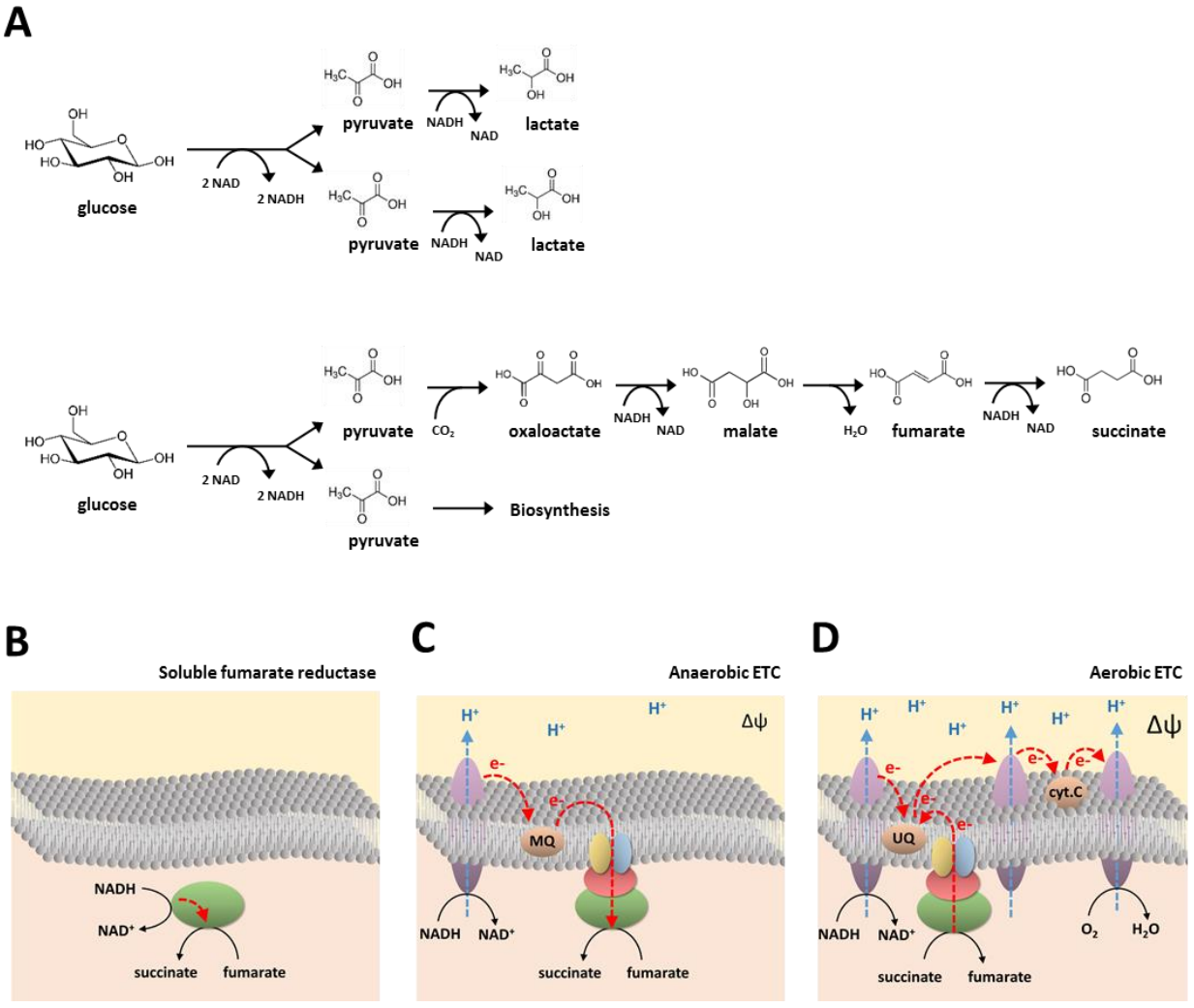

A
glucose
2 NAD
2 NADH
pyruvate
NADH
NAD
lactate
pyruvate
NADH
NAD
lactate
glucose
2 NAD
2 NADH
pyruvate
CO2
oxaloactate
NADH
NAD
malate
H2O
fumarate
NADH
NAD
succinate
pyruvate
Biosynthesis
B
Soluble fumarate reductase
NADH
NAD+
succinate
fumarate
C
Anaerobic ETC
H+
Δψ
e-
MQ
NADH
NAD+
succinate
fumarate
D
Aerobic ETC
H+
Δψ
e-
UQ
cyt.C
NADH
NAD+
O2
H2O
succinate
fumarate

Figure 5. Schematic representation of lactic and succinic fermentation and evolution on electric transport chain (ETC). (A) The lactic fermentation leads to reoxidation of only one NADH molecule by each pyruvate, while the succinic fermentation enables the reoxidation of two NADH molecules. **(B)** Soluble fumarate reductase (FRDS). The FRDS couple the reduction of fumarate to succinate with the oxidation of NADH to NAD. **(C)** Anaerobic ETC. The membrane-bound FRD couple the reduction of fumarate to succinate with the oxidation of MQH_2 to MQ, acting as a SQOR. The MQ is re-reduced by a transmembrane NADH dehydrogenase, which pumps protons from cytosol, generating a membrane potential (Δψ), which is the proton-motive force to ATP synthesis by ATP synthase. **(D)** Aerobic ETC. In aerobiosis, O_2 is the final electron acceptor of ETC and SQOR adquire SDH activity. In this context there are a larger proton pumping from cytosol, creating a larger Δψ and potentiating the ATP synthase activity. In addition, in aerobic ETC occurs the replacement of a lower reduction potential quinone (MQ) by a quinone with a higher reduction potential (UQ).

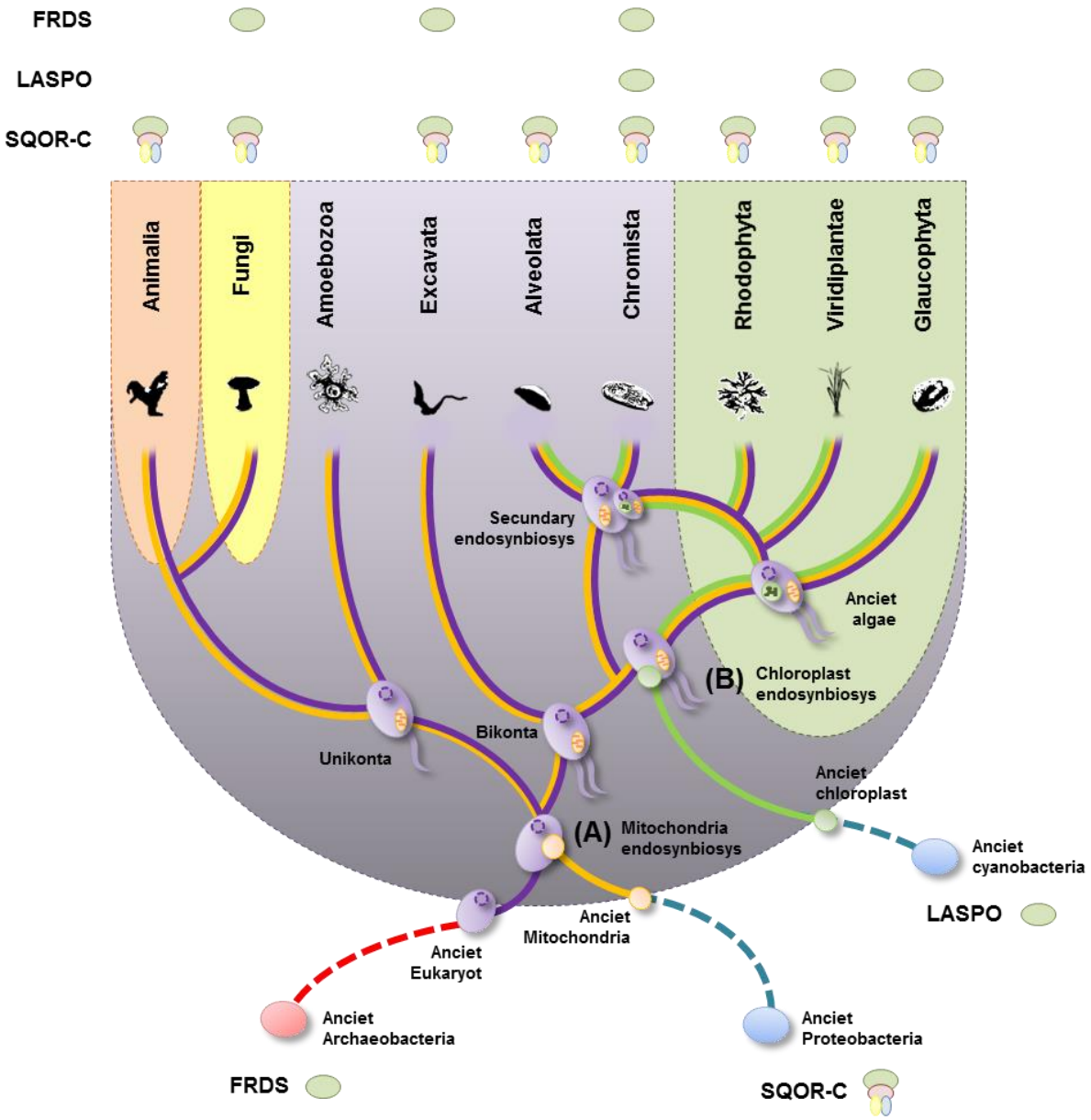

FRDS
LASPO
SQOR-C
Animalia
Fungi
Amoebozoa
Excavata
Alveolata
Chromista
Rhodophyta
Viridiplantae
Glaucophyta
Secundary endosynbiosys
Anciet algae
(B) Chloroplast endosynbiosys
Unikonta
Bikonta
Anciet chloroplast
(A) Mitochondria endosynbiosys
Anciet cyanobacteria
LASPO
Anciet Mitochondria
Anciet Eukaryot
Anciet Archaeobacteria
Anciet Proteobacteria
FRDS
SQOR-C

Figure 6. Schematic representation of life tree and mitochondrial and chloroplast endosymbiosis processes. *The dotted red line indicates Archaea and the dotted blue line indicates bacteria species. The purple lines indicate all eukaryotes, the orange lines indicate eukaryotes derived from mitochondrial endosymbiosis, and the green lines indicate eukaryotes that possess chloroplast. In addition, the eukaryotic kingdoms are indicated by background color: green to plants, yellow to fungi, pink to metazoan, and purple to all protozoan. The presence of LASPO, FRDS and SQOR type C in each eukaryotic group is indicated on the top.*

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3.3 CAPÍTULO 3

SILENCIAMENTO DA ASCORBATE PEROXIDASE ESTROMAL INDUZ TOLERÂNCIA AO ESTRESSE DE SECA EM ARROZ (*Oryza sativa L.*)

Este capítulo é referente à primeira parte do estudo funcional das isoformas cloroplastídicas de ascorbato peroxidase (APX) em arroz, e está apresentado na forma do manuscrito “THE SILENCING OF STROMAL ASCORBATE PEROXIDASE INDUCE DROUGHT STRESS TOLERANCE IN RICE (*Oryza sativa L.*)”, a ser submetido. Neste trabalho, demonstramos que plantas silenciadas para *OsAPX7* e as plantas duplamente silenciadas para *OsAPX7* e *OsAPX8* apresentaram uma maior tolerância à seca. Além disso, verificamos que o duplo silenciamento resultou em plantas com um maior conteúdo de ROS e maior fechamento estomático. Esses dados demonstram que o aumento da tolerância à seca não é consequência somente do aumento de ROS ou do fechamento estomático, uma vez que estas alterações não foram verificadas nas plantas com silenciamento simples para *OsAPX7*, as quais também apresentam um aumento na tolerância a seca. Desta forma, a isoforma *OsAPX7* estaria envovida em mecanismos ainda inéditos que permitem um aumento na tolerância a seca, sendo capazes de manter por mais tempo, parâmetros fisiológicos, como os níveis de transpiração e a fotossíntese.

THE SILENCING OF STROMAL ASCORBATE PEROXIDASE INDUCE DROUGHT STRESS TOLERANCE IN RICE (*Oryza sativa L.*)

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ABSTRACT

Ascorbate peroxidase (APX) exerts an important role in the maintenance of the level of reactive oxygen species (ROS), catalyzing the reduction of H_2O_2 to H_2O and O_2, using ascorbate as specific electron donor. In previous work was demonstrated that the double silencing of OsAPX7 (stromal APX) and OsAPX8 (thylacoidal APX), located specifically in chloroplast, led to differences in photosynthetic parameters related to efficiency of utilization of light and CO_2 in plants under stress conditions. However, the functional importance of each chloroplastic APX isoforms, and their role in plant development and in plant stress response remains unknown.

In this work, we perform a functional study of *OsAPX7* gene in rice plants, through transgenic lines, where *OsAPX7* was silenced by RNAi. In addition to RNAi*OsAPX7* plants, in this work was also used plants double silenced to *OsAPX7* and *OsAPX8* genes (RNAi*OsAPX7/8*). Our results demonstrate that the both lines show a normal growth, indicating that the silencing of chloroplastic APX do not led to changes in plants development. However, in RNAi*OsAPX7/8* plants is verified a lower chloroplastic APX activity, accompanied by a higher H_2O_2 content and increased stomatal closing. Independently of this fact, when subjected to drought stress, both transgenic lines show a higher capacity to maintain stomatal aperture and lower leaf surface temperature. Consequently, RNAiOsAPX7, as well as RNAiOsAPX7/8 plants, showed a higher tolerance to drought stress, when compared to non-transformed plants. These data indicate that the chloroplastic APX can be involved in signaling transduction pathways which regulate stomatal opening and drought stress response in rice plants.

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) are produced from the monovalent reduction of molecular oxygen (O_2), being intrinsic to the aerobic metabolism. In plants, the ROS production is induced mainly in response to adverse environmental conditions. ROS are important signaling molecules involved in key physiological pathways, such as cell cycle, programmed cell death (PCD), senescence and stress response (DAT *et al*.,2000; OROZCO-CARDENAS *et al.*, 2001; MULLINEAUX & KARPINSKI 2002; VANDENABEELE *et al*., 2003). However, at high concentration, ROS can diffuse through biological membranes reacting with biological compounds such as lipids, proteins and nucleic acids, thus promoting damage and, in many cases, cellular death (SCANDALIOS, 2002). The control of intracellular ROS levels, and consequently, the cellular redox homeostasis, is a very complex process involving many mechanisms and a large network of genes (MITTLER *et al*., 2004). The main ROS scavenging mechanism is composed by several enzymes, present in different subcellular compartments, such as, superoxide dismutases (SOD), ascorbate peroxidases (APX), catalases (CAT), glutathione peroxidases (GPx), and peroxidases (Prx) (HALLIWELL & GUTTERIDGE 1999; SCANDALIOS, 2002; MITTLER *et al*., 2004).

In plants, APX (EC, 1.11.1.11) has an important role in the ROS levels maintenance, catalyzing the reduction of H_2O_2 to H_2O and O_2, using ascorbate as specific electron donor (ASADA, 1999). In addition, APX is a component of the ascorbate-glutathione cycle, one of the most important antioxidant systems in plants. In this cycle, glutathione is used as reducing substrate to restore the ascorbate levels, and is ultimately recycled at NAD(P)H expense (ALSCHER *et al*., 1997; NOCTOR & FOYER, 1998). In rice plants (*Oryza sativa L.*), APX is encoded by a small gene family, in which the isoforms are targeted to distinct subcellular compartments: cytosol (*OsAPX1* and *OsAPX2*), peroxisome

(*OsAPX3* and *OsAPX4*), mitochondrial (*OsAPX5* and *OsAPX6*) and chloroplast (*OsAPX5*, *OsAPX7* and *OsAPX8*) (TEIXEIRA *et al*., 2006).

Our group has demonstrated the functional importance of the different APX isoforms in the cellular homeostasis in rice. Recently, we demonstrated that rice plants silenced in both exclusive chloroplastic APXs (*OsAPX7* and *OsAPX8*) exhibited differences in photosynthetic parameters related to efficiency of utilization of light and CO_2 when submitted to stress conditions, suggesting that a partial deficiency of APX in chloroplast modulate the photosystem activity and integrity mainly under abiotic stress conditions. In addition, *OsAPX7* and *OsAPX8* have an antagonic expression pattern under several stress conditions, being induced and repressed, respectively (CAVERZAN *et al*.,2012). This data suggests that chloroplastic APXs exert a different role in stress response, however, the individual contribution of each chloroplastic APX in normal and in stress conditions remains unknown.

In this work, we performed a functional study of *OsAPX7* gene using rice plants silenced to *OsAPX7* (RNAi*OsAPX7*) and plants double silenced to *OsAPX7* and *OsAPX8* genes (RNAi*OsAPX7/8*). These transgenic plants have a growth similar than non-transformed (NT) plants, however, the RNAiOsAPX7/8 plants show lower chloroplastic APX activity and higher H_2O_2 release, which can explain the decrease of stomata opening. On the other hand, when subjected to drought stress, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants show a higher capacity to maintain stomatal aperture and lower leaf surface temperature, resulting in a higher tolerance to drought stress, as compared to NT plants. These results demonstrate that the silencing of *OsAPX7* do not change the redox homeostasis in chloroplast, but increase the tolerance to drought stress, indicating that *OsAPX7* could be involved in stress signaling pathway. In addition, this work demonstrate that only OsAPX7 silencing is necessary to improve drought tolerance in rice.

MATERIAL AND METHODS

Plant material and growth conditions

Rice (*Oryza sativa* L. japonica cv. Nipponbare) seeds were germinated in MS medium (Sigma-Aldrich) at 25 °C with a 12-h photoperiod. One-week-old rice seedlings were transferred for hydropony in 200-ml plastic cups (three seedlings per cup) filled with Hoagland–Arnon's nutritive solution (Hoagland & ARNON, 1950). To drought experiments, the one-week-old rice seedlings were transferred to soil in plastic vessels. Was used a mixture of soil, vermiculite and peat (2:1:1) and was placed six plants per vessel. The drought experiment was performed in one-month-old plants, thought the suspense of watering. The drought stress was maintained until the relative water content in vessel reaches 10% (in about eight days). The control of relative water content during drought stress experiment is showed in Supplementary figure S1. To ABA treatment, 100µM ABA was dissolved in 0.1% Triton-X-100 and sprayed in excess on the leaves of the plants until complete wetting was achieved.

Vector construction and plant transformation

A plasmid was constructed to express a hairpin structure (hpRNA) based on the sequence of *OsAPX7* (LOC Os04g35520) gene and was named RNAi*OsAPX7*. The following primers were used to amplify a 220 bp fragment from the *OsAPX7* gene to clone into the RNAi vector: 5'-CTCCGAGCAATCTGGGTGCAAAAAT-3' and 5'-GGTACCTCGAGGACTCGTGGTCAGGAAAAGC-3'. The PCR product was cloned into the Gateway RNAi vector pANDA, in which the expression of the

hairpin RNA is driven by the maize ubiquitin promoter (MIKI & SHIMAMOTO, 2004). *Agrobacterium tumefaciens*-mediated transformation was performed as previously described (UPADHYAYA *et al*., 2002).

To make a translational fusion of the OsAPX7 protein with Yellow Fluorescent Protein (YFP) on the N-terminus and HA on the C-terminus, the *OsAPX7* (LOC_Os04g35520) gene was amplified from rice leaf cDNA using the primers 5′-ATGGCGGCCCAGCG-3′ and 5′-ACCGTCCAACGTGAATCC-3′ and cloned into the pART7-HA-YFP plasmid (GALVÁN-AMPUDIA & OFFRINGA, 2007). The gene expression is under control of 35S promoter of CaMV. The construct was then used to transform rice protoplasts.

To verify the *OsAPX7* promoter expression pattern, a 2 kb fragment upstream of the start codon was amplified by PCR, cloned into pENTR (Invitrogen®) and recombined with pHGWFS7 (KARIMI *et al*., 2005) producing the vector promAPX7-GUS. The promAPX7-GUS vector contains the Gus and GFP as reporter genes and *hpt* as selection marker gene that confers plant resistance to hygromycin. The following primers were used to amplify the *OsAPX7* promoter: 5′-TGATAGAGTTAGTTTATGTCAAACTCG-3′ and 5′-GACGAGGCGGAGTGAGGT 3′. The plasmid was transformed into rice calli via *Agrobacterium tumefaciens*-mediated transformation, as described by UPADHYAYA *et al*. (2002).

Isolation and transformation of protoplasts

Protoplast isolation was performed as described by CHEN *et al*., (2006) and protoplast transformation as described by TAO *et al*., (2002). After transformation, protoplasts were incubated for 24–48 h in the dark at 28 °C before imaging. Fluorescence was monitored using an Olympus FluoView 1000 confocal laser scanning microscope (Olympus, Japan) equipped with a set of filters capable of distinguishing between green and yellow fluorescente protein (GFP and YFP, respectively) and plastid autofluorescence. The images were captured with a high-sensitivity photomultiplier tube detector.

Analysis of the *OsAPX7* promoter expression pattern in rice plants

Leaves, roots, stem and panicles of transgenic rice plants expressing Gus gene under the control of the *OsAPX7* promoter (promAPX7-GUS) were sampled and analyzed using the X-Gluc histochemical assay (Fermentas®), as described in JEFFERSON *et al*., (1987), with minor modifications. After Gus staining, the samples were clarified with graded ethanol series (30–70%) and analyzed in stereomicroscope and bright field microscopy.

Methyl violagen (MV) treatment

The one-month-old plants were grown as previously described. The 50 µM MV was prepared in 0.1% Triton-X-100 and sprayed in excess on the leaves of the plants until complete wetting. 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 conditions as the plants submitted to the MV treatment.

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen, Waltham, MA, USA). Complementary cDNA was obtained using the SuperscriptTMII (Life Technologies, Carlsbad, CA, USA) reverse transcriptase system and a 24-polyTV primer (Invitrogen®). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method (LIVAK & SCHMITTGEN, 2001; SCHMITTGEN & LIVAK, 2008). RT-qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Biosystems, Waltham, MA, USA) using SYBR-green intercalating dye for fluorescence

detection. The primer sequences and reference genes are listed in Supporting Information Table S1.

Quantitative measurement of H_2O_2

The measurement of H_2O_2 production was performed by extracting H_2O_2 from leaves according to a previously described method (RAO *et al*., 2000) using the Ampliflu Red (Sigma-Aldrich) oxidation method as previously described (SMITH *et al*.,2004). Fluorescence was monitored using a fluorimeter at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H_2O_2.

Meansurement of chloroplastic APX activity

The chloroplast was isolated from one-month-old plants as SEIGNEURIN-BERNY *et al*. (2008), and APX activity was measured as CAVERZAN *et al*. (2012).

Pigment determination

The total chlorophyll, chlorophyll a and b were determined after extraction in ethanol and measured spectrophotometrically at 665 and 649 nm. The amount of pigment was calculated using the equations proposed by LICHTENTHALER & WELLBURN (1983). In addition, the pigment determination was performed by co-focal microscopy, using the red filter of chlorophyll.

Membrane damage determination

The electrolyte leakage (membrane damage) was measured as described previously by BLUM & EBERCON (1981). Leaf samples were placed in tubes containing deionized water. The flasks were incubated in a shaker for 12 h, and the electric conductivity in the medium (L1) was measured. Then, the medium was boiled (95 °C) for 60 min and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2×100.

Imaging of rice stomata

The stomatas were visualized by scanning electron microscopy and quantified. Leaves of 30 day-old plants from different height were detached and the samples are prepared as described by HUANG *et al*., (2009).

Gas exchange and photochemical parameters

The following PSII parameters were measured using an Infrared Gas Analyzer coupled with a leaf chamber fluorometer (Li-6400-XT, LI-COR, Lincoln, USA) according to the manufacturer's instructions. $\Delta F/F'_m$, 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 = (ΔF/F'm× PPFD × 0.5 × 0.84), where 0.5 is the presumed fraction of the excitation energy distributed to PSII and0.84 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 [$qP = (F'_m - F_s)/(F'_m - F'_o)$] and the non-photochemical quenching coefficient [$NPQ =(F_m -$

$F'_m)/F'_m]$, where F_m and F_o are, respectively, maximum and minimum fluorescence of dark-adapted leaves; F'm and F_s are, respectively, maximum and steady state fluorescence in the light-adapted state, and F'_o 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-redGas Analyzer (Li-6400-XT, LI-COR Biosciences). 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 ºC and relative humidity of 78% and vapor pressure deficit (VPD) of 1.8 Pa. The gas exchange variables measured were net photosynthesis (PN) and intercellular CO_2 concentration (CI).

Thermal Imaging

Thermography was performed using a ThermaCam B20HS camera (FLIR Systems) equipped with an uncooled 320 x 240 microbolometer matrix detector in the 7- to 13-mm band having an improved sensitivity (noise-equivalent differential temperature below 0.05°C). These subsequent experiments were carried out in a dedicated darkroom under low wind speed to ensure temperature contrast between lines.

Protein determination

Protein concentrations were determined as described by BRADFORD (1976) using BSA as a standard.

Statistical analysis

Data were plotted with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and analyzed by one-way ANOVA and a posteriori Tukey's test. P-values of 0.05 were considered statistically significant.

RESULTS

OsAPX7 protein is localized in chloroplast and *OsAPX7* gene mainly expressed in embryo and vascular tissue

In a previous work, eight *APX* genes were identified in the rice genome (TEIXEIRA *et al*., 2006). *In silico* analysis predicted that the protein encoded by *OsAPX7* is exclusively located in chloroplast. In the present work, the subcellular localization of OsAPX7 protein was experimentally determined in rice protoplasts. We constructed a translational fusion of OsAPX7 with YFP protein, driven by the CaMV 35S promoter. Confocal analysis of protoplasts expressing 35S-OsAPX7::YFP fusion revealed that OsAPX7 was localized in the chloroplast (Fig. 1A). These data confirmed the previous *in silico* prediction, which indicated that OsAPX7 was located in stroma (TEIXEIRA *et al*., 2006).

To analyse the *OsAPX7* gene expression pattern, rice calli were transformed with the promAPX7-Gus construct and then we analyzed leaves, roots, stems, and spikelets from two different transgenic rice lines (proAPX7-Gus). This analysis showed Gus staining in the phloem region of the stem (Fig. 1G and H), in tip lemma/palea of spikelet (Fig. 1I), in the embryo of a fully expanded rice seed (Fig. 1J), and in young trichomes of rice spikelets (Fig. 1K).

Molecular and morphological analysis of *OsAPX7* silenced plants

To determine the functional role of the stromal ascorbate peroxidase, the *OsAPX7* gene was silenced using the RNAi technology to generate the transgenic rice lines, named RNAi*OsAPX7*. In addition, in this work, we used plants that have are both chloroplast APX genes (*OsAPX7* and *OsAPX8*) silenced. These transgenic plants were previously obtained by CAVERZAN *et al*., (2012) and named RNAiOsAPX7/8.

The RT-qPCR analysis of RNAi*OsAPX7* T2 plants showed that *OsAPX7* transcript levels were reduced in two lines, which were names as line-a and line-b. In these lines the OsAPX7 transcript was reduced to 71% and 76%, respectively, as compared to NT plants. The double silenced plants (RNAiOsAPX7/8) showed a decrease of about 50% for *OsAPX7* transcript level and 60% for *OsAPX8*.

RNAi*OsAPX7* plants did not show an altered *OsAPX8* transcript levels (Fig. 2G and 2H). On the other hand, in both RNAi*OsAPX7* lines, the expression of *OsAPX2* was also reduced, in about 60% (Fig. 2B). In addition, these plants showed an increase of about 6 and 4-fold in the expression of *OsAPX6* (Fig. 2F). To investigate the effect of *OsAPX7* silencing in plant development, the growth of RNAi*OsAPX7* and RNAi*OsAPX7/8* plants was analyzed. Our results demonstrated that, under normal conditions, the transgenic plants did not show apparent changes in growth when compared with non-transformed plants grown under control conditions. Thus, all plants showed the same leaf length and growth rate (Fig. 3). In the future experiments, the RNAi *OsAPX7* line-a and RNAi *OsAPX7/8* line-a plants was used as representative lines.

Effect of RNAi*OsAPX7* silencing in chloroplastic APX activity and in response to MV treatment

To analyze the effect of *OsAPX7* silencing in chloroplastic APX activity, we measured the APX activity in isolated chloroplast. The APX activity was measured using different concentration of H_2O_2. Our results demonstrate that the *OsAPX7* silencing do not change APX activity (Fig. 4A). On the other hand, the double silencing led to a decrease of APX activity (Fig. 4B). In addition, we determined different catalytic parameters of chloroplastic APX activity in NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants. In both transgenic plants lines there are not changes in V_{MAX} (Fig. 4C), however in RNAi*OsAPX7/8* plants is verify an increase in about three fold in apparent K_M to H2O2 (Fig. 4D), which results in an

equivalent decrease of catalytic efficiency of chloroplastic APX activity (V_{MAX}/K_M) (Fig. 4E).

In addition, to verify the effect of APX silencing in chloroplast stress response, we evaluated the change of chlorophyll content in leaves treated with methyl viologen (MV), which is able to induce ROS production in the chloroplast. No differences were verified between NT and RNAi OsAPX7 plants, however, double-silenced plants showed a higher decrease of chlorophyll content after MV treatment, however, under control conditions no differences in the chlorophyll content was observed (Fig. 5A-5C).

RNAi*OsAPX7* silenced plants show improved drought tolerance

It has been demonstrated that control of ROS production and scavenging in the chloroplast are essential for drought tolerance in both transgenic plants and tolerant cultivars (VAN CAMP *et al.*, 1996; HERNANDEZ *et a.l*, 2001; MITTLER & BERKOWITZ, 2001; TSENG *et al.*, 2007). To evaluate the effect of the *OsAPX7* silencing on drought tolerance, we performed a soil drought experiment. Fig. 6A and 6B show that both RNAi*OsAPX7* and RNAi*OsAPX7/8* plants have higher drought tolerance when compared to NT plants. In addition, we repeat the drought experiment using an additional line of RNAi*OsAPX7*. Our results demonstrate that both lines (lina-a and line-b) show an increased tolerance to drought stress (Supplementary figure S2).

To evaluate the cell membrane damage in leaves of transgenic and NT plants subjected to drought stress, we have determined the electrolyte leakage. Under drought stress, both RNAi*OsAPX7* and RNAi*OsAPX7/8* showed lower electrolyte leakage under drought stress, as compared to NT plants (Fig. 6C). These results indicated that silencing of the *OsAPX7* gene prevents cell membrane damage under drought stress, thus contributing to improve drought tolerance.

To elucidate the physiological mechanism of drought tolerance in RNAi*OsAPX7* silenced plants, we compared gas exchange and photochemical parameters between NT and silenced plants under drought stress condition. NT plants showed a decrease of apparent transpiration (E), net photosynthesis (Pn), photosystem II activity (Y) and electron transport rate (ETR) after the fourth day of drought, while silenced plants maintain the values for these parameters, being affected by drought stress only at the fifth day (Fig. 7A, 7C, 7D, 7E, respectively). Consequently, the RNAi*OsAPX7* plants show an increased internal CO_2 concentration in a later moment than NT pants (Fig. 7B). These results indicate that the enhanced drought tolerance of the RNAi*OsAPX7* silenced plants is due to an increased ability to maintain water use efficiency (WUS) and the potential quantum yield of photosystem II (Fv/Fm) under drought stress condition (Fig. 7F and 7G, respectively).

The *OsAPX7* silencing causes decrease of stomatal opening

Due the importance of stomata in the responses to drought (HETHERINGTON & WOODWARD 2003), we examined the stomata status in RNAi*OsAPX7* plants and RNAi*OsAPX7/8* plants under control conditions. Our results showed that 29.0% and 49.6% of the leaf stomata were completely closed in the RNAi*OsAPX7* silenced plants and in the RNAi*OsAPX7/8* plants, respectively, while only 20,3 % were completely closed in NT plants. In addition, 22.1% of stomata were completely open in RNAi*OsAPX7* and only 0.8% in RNAi*OsAPX7/8* plants, while 39.9% of stomata were completely open in NT plants. The percentage of partially open stomata was 49.6%, 48.8% and 38.8% in NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants, respectively (Fig. 8B). These results showed that *OsAPX7* silencing do not changes the stomatal opening pattern, however the double silencing of *OsAPX7* and *OsAPX8* led to a decreased stomatal opening, indicating that stomata aperture can be greatly

affected by the silencing of both chloroplastic APX in rice plants. Previous work has demonstrated the ROS involvement in stomata closure, mainly in response to drought stress (DASZKOWSKA-GOLEC & SZAREJKO, 2013). Thus, we measured the hydrogen peroxide in transgenic and NT plant leaves. Our results showed an increase in about 25% in hydrogen peroxide in RNAi*OsAPX7/8* plants, while no differences were observed in the hydrogen peroxide content between NT and RNAi*OsAPX7* plants (Fig. 8C).

In addition, to analyze the effect of *OsAPX7* silencing in leaf water loss, we also subjected rice leaves to a desiccation experiment. Our results demonstrated that *OsAPX7* silencing do not change leaf desiccation, however leaves from RNAi*OsAPX7/8* plants showed a reduction of about 25% in desiccation as compared to NT plants, consistent with the results observed for stomatal aperture. Interestingly, the pre-treatment with abscisic acid led to a decrease of leaf desiccation in NT and in RNAi*OsAPX7* plants, and do not change the leaf desiccation in double knockdown. Under this condition, there are not differences in leaf desiccation between NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants (Fig. 8D-8F). These results indicate that the silencing of *OsAPX7* led to decrease of stomatal opening but not changes leaf desiccation.

The *OsAPX7* silencing led to decrease of leaf surface temperature even in drought stress condition

An alternative approach for measuring stomatal conductance is the infrared (IR) thermography in leaves. The leaf surface temperature correlates well with estimates of stomatal conductance (JONES, 1999; SIRAULT *et al*., 2009) and consequently with rice water status (CAO *et al*., 2013). Thus, we measured the leave surface temperature in NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants under control conditions, moderate drought stress (2 days water withholding) and severe drought stress (7 days water withholding). Our results showed that under control conditions, RNAi*OsAPX7* silenced plants have about 0.85 ºC decrease in

leaf surface temperature (Fig. 9A). Under severe drought stress, the RNAi*OsAPX7* silenced plants remains with a lower leaf surface temperature, -0.61 ºC and -0.34 ºC, respectively (Fig. 9B and 9C). On the other hand, the RNAi*OsAPX7*/8 plants did not show an altered leaf surface temperature in either control or stress conditions. These results indicate that the silencing of *OsAPX7* led to a decrease of leaf surface temperature, which remains lower even under drought stress condition.

DISCUSSION

The subcellular localization of all OsAPX family members was previously predicted by *in silico* analysis (TEIXEIRA *et al*., 2006). In the present study, our data confirmed the predicted subcellular localization of OsAPX7 protein, which is located in the chloroplasts of rice protoplasts. In addition, the analysis of the *OsAPX7* gene promoter demonstrated that *OsAPX7* gene is expressed predominantly in the embryo, phloem region of stem, tip and young trichrome of rice spikelet. In fact, H_2O_2 has been recognized as a phloem-mobile signal, which moves from the local tissue to the entire plant (SZECHYNSKA-HEBDA *et al*., 2010; SUZUKI *et al*., 2013; GILROY *et al*., 2014). In *Arabidopsis thaliana*, using Luciferase gene expression driven by the H_2O_2 inducible APX2 promoter, was demonstrated that H_2O_2 have a crucial role in systemic acquired acclimation (SAA) in response to high light stress (KARPINSKI *et al*., 1999). Thus, the presence of APX in the vascular cells could regulate local or systemic ROS signaling.

We have previously demonstrated that the double silencing of chloroplastic APXs (*OsAPX7* and *OsAPX8*) does not result in visible growth changes of plants under control growing conditions, but it causes alterations in the level of proteins involved in photosynthesis and oxidative metabolism. Moreover, under abiotic stress conditions, the double knockdown plants showed many photosynthetic and biochemical changes (CAVERZAN *et al*., 2012). Although this work has reported the importance of OsAPX7 and APX8 isoforms in abiotic stress response in rice plants, the particular role of each APX isoforms remained unknown. Thus, here we conducted a functional study of OsAPX7, showing its importance in plant development and in the control of drought stress response, through the analysis of RNAi*OsAPX7* plants.

Our results indicate that, in control conditions, the decrease in about 90% of the *OsAPX7* transcript did not impair chloroplastic APX activity and the normal development of rice plants. In addition, these plants did not exhibit symptoms of oxidative stress, as membrane damage or reduced growth. In

contrast, the RNAi*OsAPX7/8* plants, which have both chloroplastic APXs silenced, showed a decrease of chloroplastic APX activity and a slight increase of hydrogen peroxide content in leaves when compared to NT plants, as demonstrated previously by CAVERZAN *et al*., 2012.

As described by DASZKOWSKA-GOLEC & SZAREJKO (2013) the increase of ROS production is involved with different stomata closure mechanisms. Thus, the RNAi*OsAPX7/8* plants showed a decrease of stomatal opening and, consequently, decrease of leaves desiccation. These phenomena can be justified by an increase of hydrogen peroxide content in leaves of RNAi*OsAPX7/8* plants.

Interestingly, our group demonstrated in a previous work that *OsAPX7* expression is highly induced under chloroplast stress condition, as high light or MV treatment (CAVERZAN *et al*., 2012). These results indicate that OsAPX7 can be involved in the chloroplast stress response mechanisms. Due to the importance of the regulation of ROS production and scavenging by chloroplast in plant tolerance to drought (VAN CAMP *et al*., 1996; HERNANDEZ *et al*., 2001; MITTLER & BERKOWITZ, 2001; TSENG *et al*., 2007), we subjected the RNAi*OsAPX7* and RNAi*OsAPX7/8* plants to drought stress, to determine the physiologic role of chloroplastic *OsAPX7* in drought stress response. Surprisingly, both transgenic plants show an increased drought tolerance when compared to NT plants. To understand the mechanism of drought tolerance in these transgenic plants, we evaluated photochemical parameters and gas exchange in plants exposed to drought stress. Our results revealed that RNAi*OsAPX7* and RNAi*OsAPX7/8*plants are able to maintain the transpiration, and consequently stomatal conductance, for a longer time under drought stress. The capacity to maintain the stomatal conductance under stress condition enables the maintenance of photosynthesis, resulting in a later increase in intracellular CO_2 concentration. Consequently, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants are able to maintain for a longer time the water use efficiency and the potential quantum yield of photosystem II under drought stress condition. This later effect of drought stress on photosynthesis suggests that the silencing of OsAPX7 led to a prevention of inhibition of Calvin cycle biochemical

reactions, due the ability of transformed plants to maintain the stomatal conductance and the CO_2 assimilation, even under drought stress. The maintenance of the Calvin cycle, therefore, enables continuous oxidation of NADPH to NADP, which is required as the final electron acceptor of photosynthetic electron transporter chain activity.

In addition, we established the rice water status by leaf canopy temperature in plants subjected or not to drought stress. Surprisingly, the RNAi*OsAPX7* plants showed a decrease of about 0.85 ºC in the leaf surface temperature. This decrease was maintained even under moderate or severe drought stress. At first, this result may seem contradictory, since the RNAi*OsAPX7* plants do not show an increase of stomatal opening or transpiration. However, the rice water status is not only dependent of transpiration, but is also highly dependent of water absorption. Thus, the decrease of leaf surface temperature in RNAi*OsAPX7* plants can indicates an increase of water absorption capacity and new studies should be conducted to test this hypothesis. In fact, lines more productive (and tolerant) to drought stress condition, especially crops, has been selected based on their lower leaf temperature. Previous work, in wheat and maize, demonstrate that cooler leaf surface temperature is favorable for higher yield (JONES, 1999). The higher transpiration in stress condition indicates a higher stomatal conductance, and therefore, greater capacities of plants to continue to perform photosynthesis and being potentially more productive. In potato, for example, it was demonstrated a clear negative correlation between the temperature of the canopy and the production of tubers, when cultivated under large moisture supply (PRASHAR *et al*., 2014). However, this strategy can be dangerous. A higher stomatal conductance under stress condition enable a higher photosynthesis, but the water loss by transpiration is also increased. This can be serious in severe and prolonged stress situations, which could compromise not only the production but also the plant survival. This work demonstrates for the first time the functional study of OsAPX7, and that its manipulation led to rice plants more tolerant to drought stress. More than a biotechnological application, this work reveals the

complexity of the control exercised by the organelle ROS production in the signaling transduction pathways which regulate the abiotic stresses response.

FIGURES

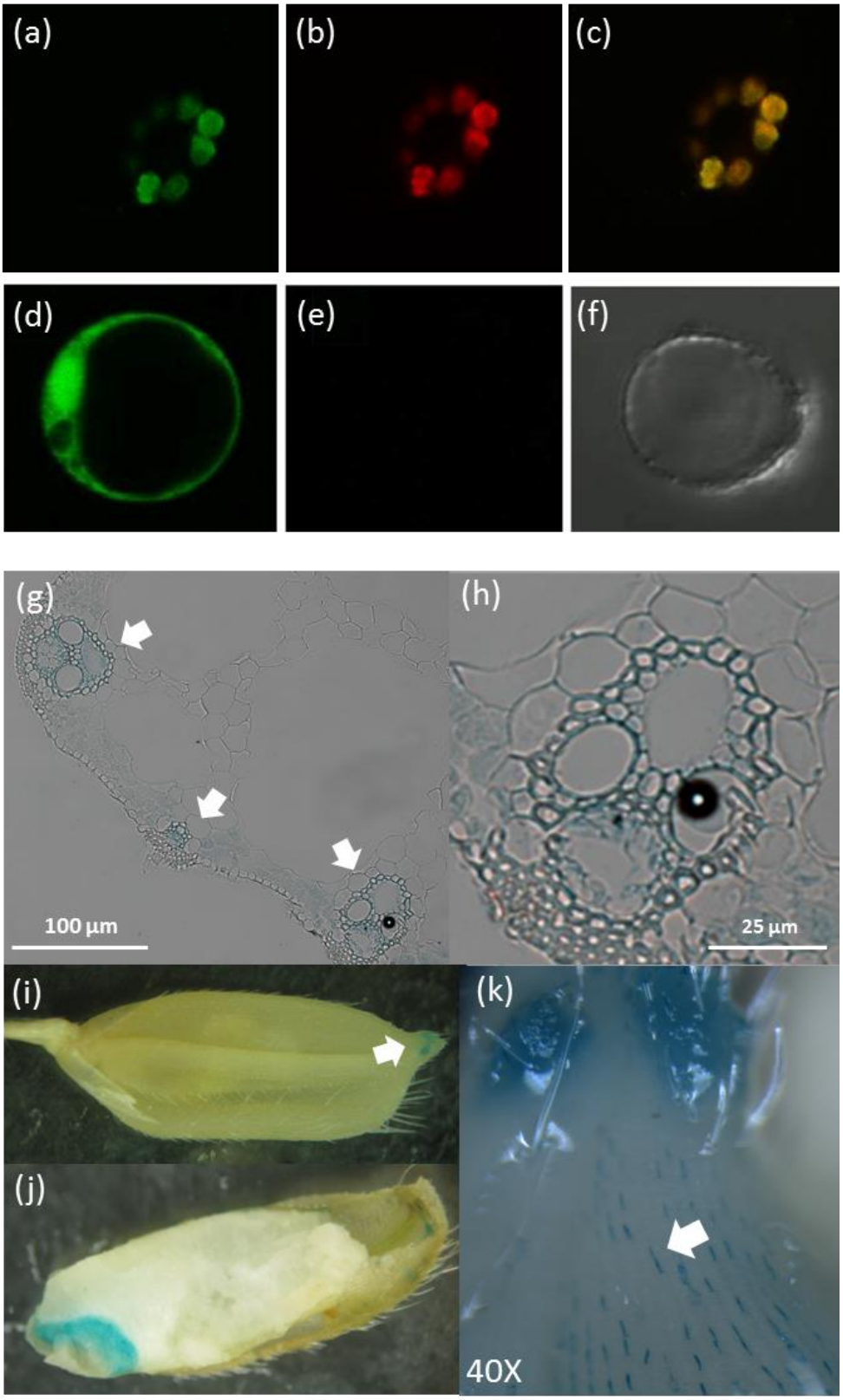

Figure 1. Subcellular localization of OsAPX7 protein and analysis of expression patterns of *OsAPX7* promoter merged to the Gus sequence. Subcellular localization of the OsAPX7 protein in chloroplasts in rice protoplasts throught transient expression of the 35S-OsAPX7::YFP cassette. **(a)** green signals indicate YFP fluorescence; **(b)** red signals indicate chlorophyll autofluorescence and **(c)** yellow signlas is the merged image; **(d)** positive control of the location using 35S::YFP cassette; **(e and f)** negative control; Expression patterns of *OsAPX7* promoter gene merged to the Gus sequence. **(g and h)** six-month-old rice plants: phloem of stem; **(i)** tip lemma/palea of spikelet; **(j)** embryo of a fully expanded rice seed and **(k)** young trichomes of rice spikelet.

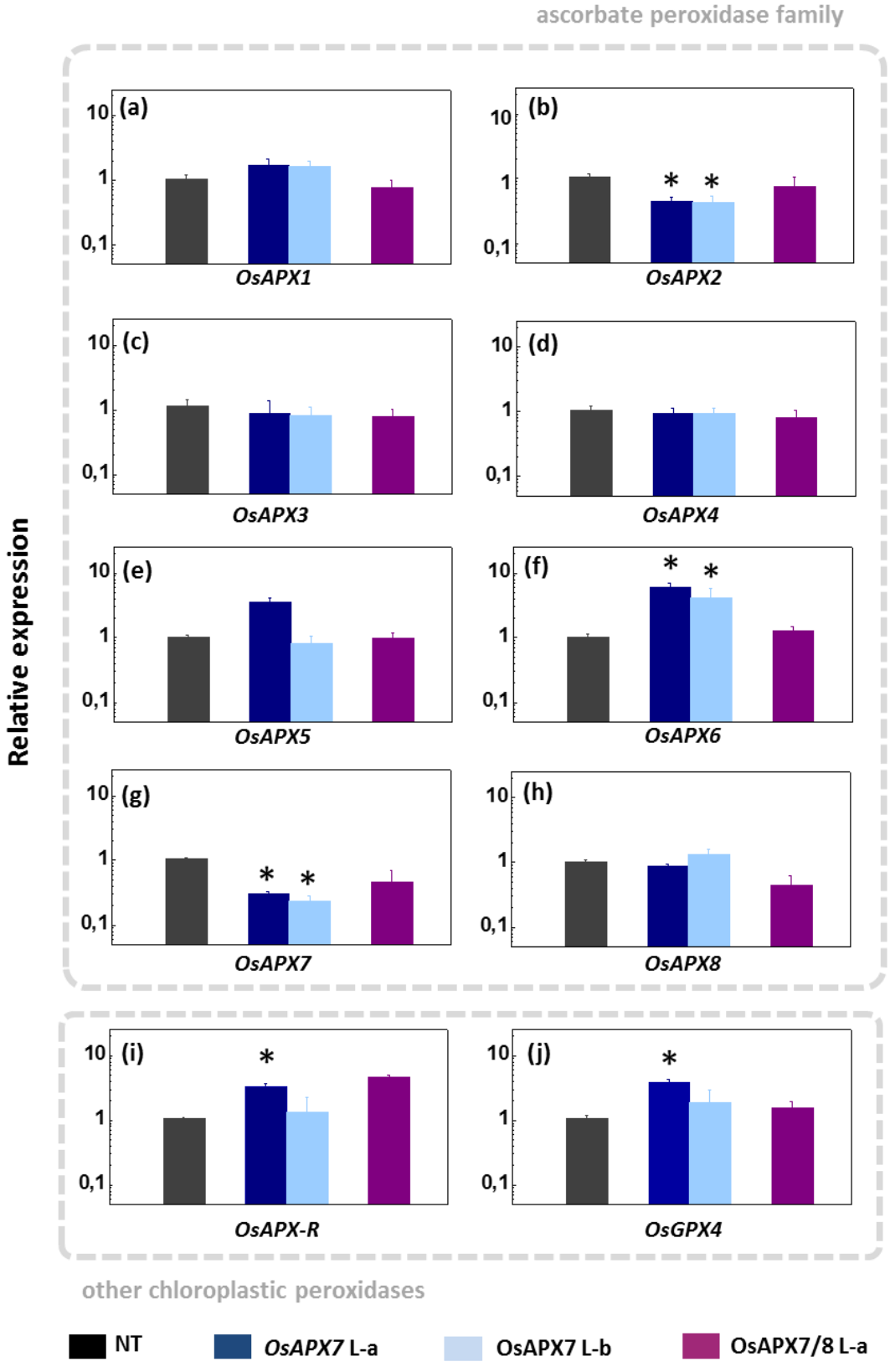
ascorbate peroxidase family
Relative expression
(a)
OsAPX1
(b)
OsAPX2
(c)
OsAPX3
(d)
OsAPX4
(e)
OsAPX5
(f)
OsAPX6
(g)
OsAPX7
(h)
OsAPX8
(i)
OsAPX-R
(j)
OsGPX4
10
1
0,1
other chloroplastic peroxidases
NT
OsAPX7 L-a
OsAPX7 L-b
OsAPX7/8 L-a

Figure 2. Quantitative determination of ascorbate peroxidase family and other chloroplastic peroxidases mRNA in leaves of NT (non-transformed), RNAi*OsAPX7* and RNAi*OsAPX7/8* plants. The values were normalized by the at least three constitutive genes and represent the media ± SE of at least three independent experiments. (*) indicate populations significantly different with $p<0,05$.

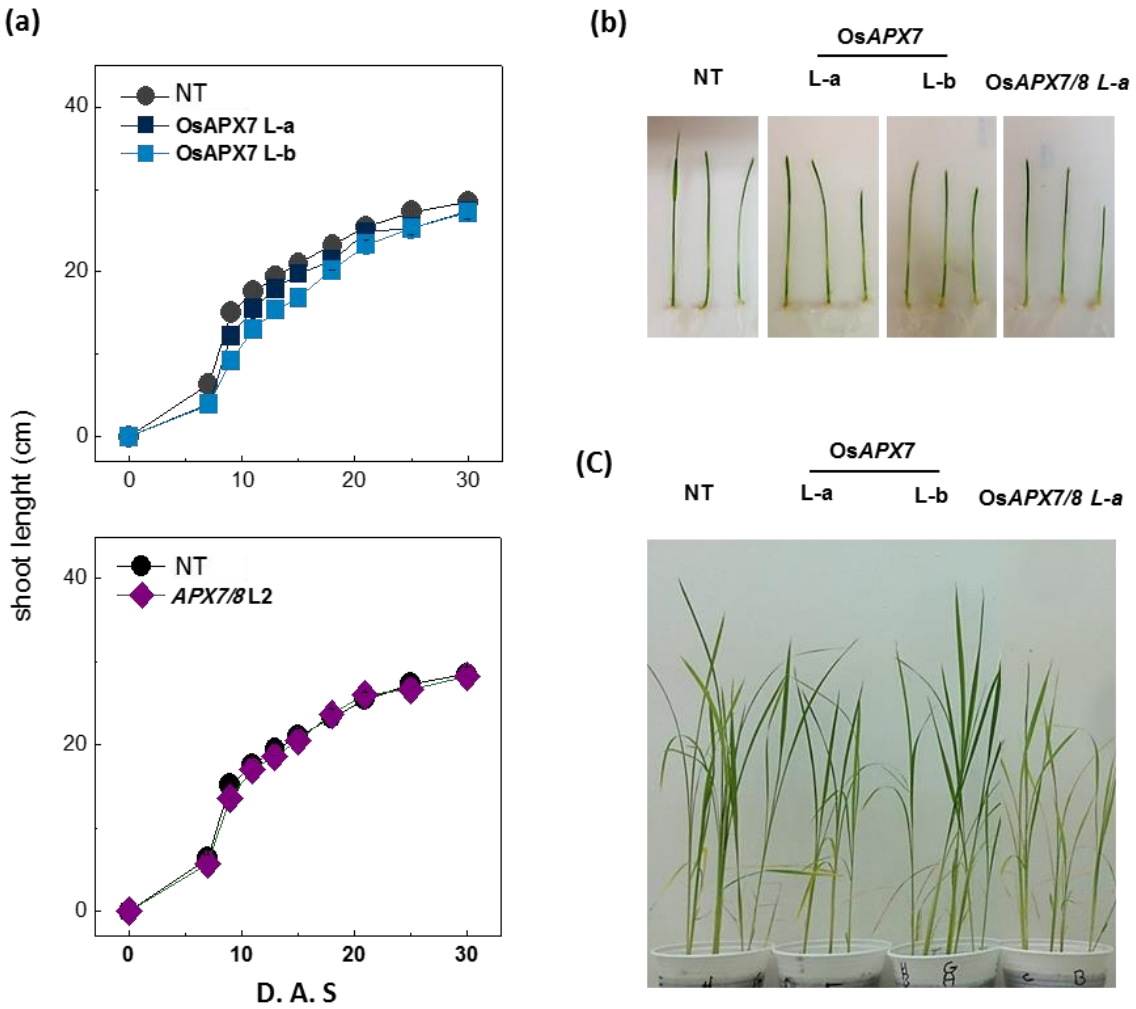

Figure 3. Growth characterization of NT (non-transformed), RNAi*OsAPX7* and RNAi*OsAPX7/8* plants. (a) Growing curve of NT (grey), RNAi*OsAPX7* plants (dark and light blue) and RNAi*OsAPX7/8* plants (purple). **(b)** NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* seedlings with seven days old. **(c)** NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants with four weeks old.

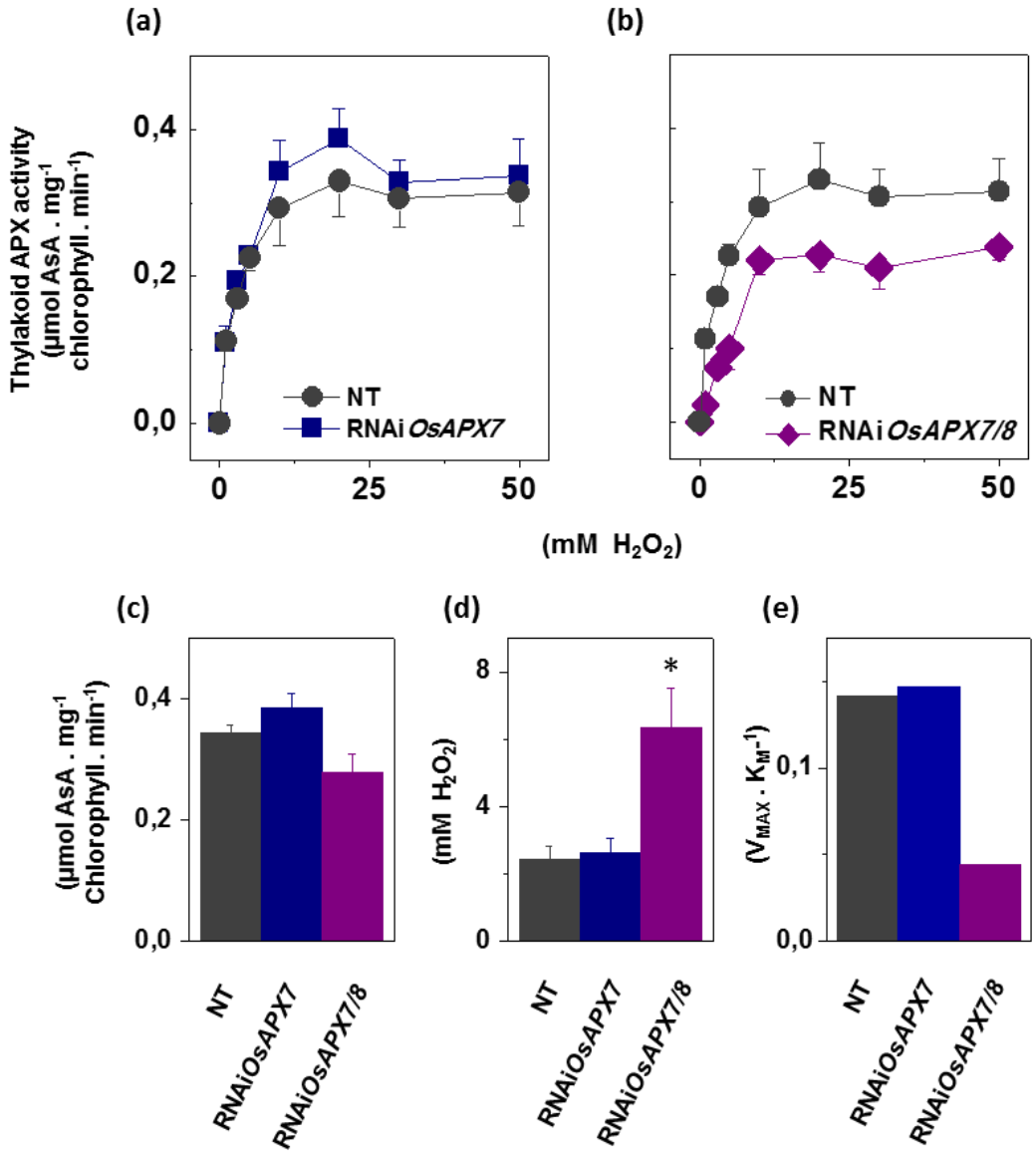

Figure 4. Chloroplastic APX activity in NT, RNAiOsAPX7 and RNAiOsAPX7/8 plants. (a) and **(b)** Measurement of APX activity under different H_2O_2 concentration in isolated chloroplast from NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants leaves. Kinetics parameters of APX activity: **(c)** V_{MAX}, **(d)** K_M and **(e)** catalytic efficiency (V_{MAX}/K_M). (*) indicate populations significantly different with p<0,05.

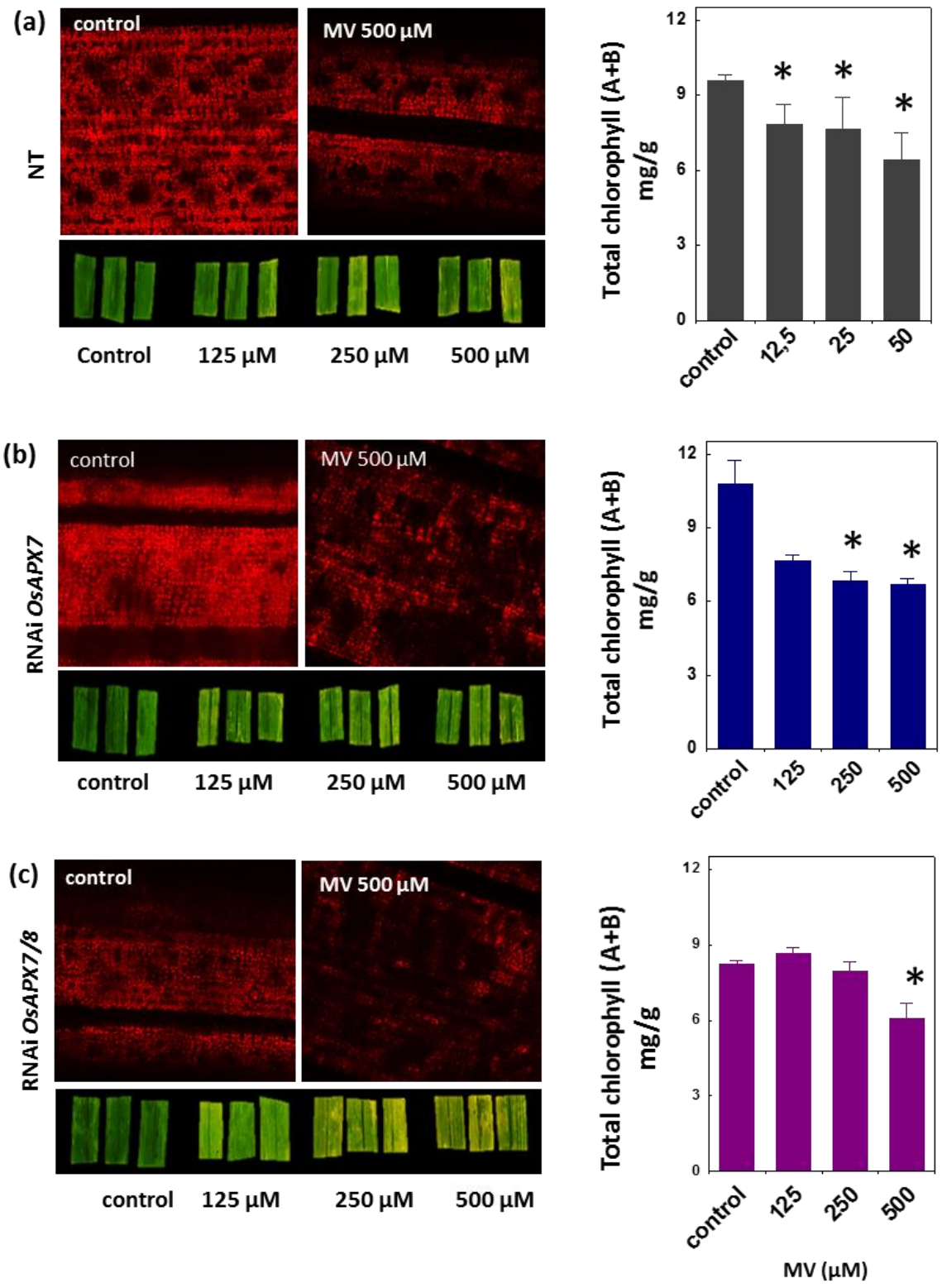
(a)
NT
control
MV 500 µM
Control
125 µM
250 µM
500 µM
Total chlorophyll (A+B) mg/g
12
9
6
3
0
*
*
*
control
12,5
25
50
(b)
RNAi OsAPX7
control
MV 500 µM
control
125 µM
250 µM
500 µM
Total chlorophyll (A+B) mg/g
12
9
6
3
0
*
*
control
125
250
500
(c)
RNAi OsAPX7/8
control
MV 500 µM
control
125 µM
250 µM
500 µM
Total chlorophyll (A+B) mg/g
12
9
6
3
0
*
control
125
250
500
MV (µM)

Figure 5. Effect of MV treatment in chlorophyll content of NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants. The chlorophyll content was evaluated by co-focal microscopy (left) and quantified by colorimetric method (right) in leaves from NT **(a)**, RNAi*OsAPX7* **(b)** and RNAi*OsAPX7/8* plants **(c)**. (*) indicate populations significantly different with p<0,05.

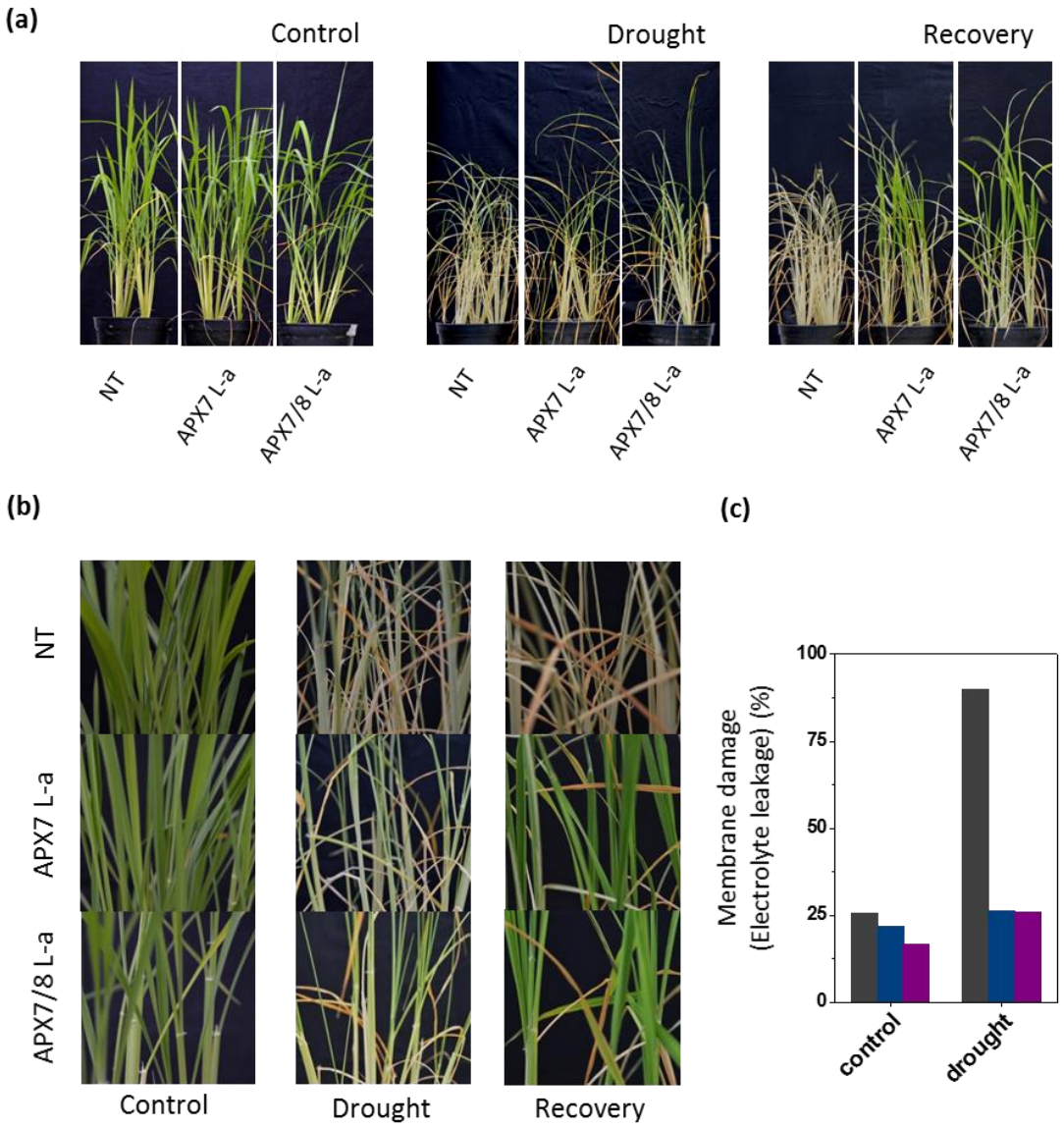

Figure 6. Effect of knockdown of chloroplastic APX in drought stress tolerance. **(a)** Drought stress treatment in NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants. One mouth-old plants (left panel) were submitted to eight day of drought (middle panel) and then then recovered by three days (right panel). **(b)** Closer pictures of plants shown in figure **(a)**. **(c)** Membrane damage of NT (grey), RNAi*OsAPX7* (blue) and RNAi*OsAPX7/8* plants (purple) submitted to drought stress.

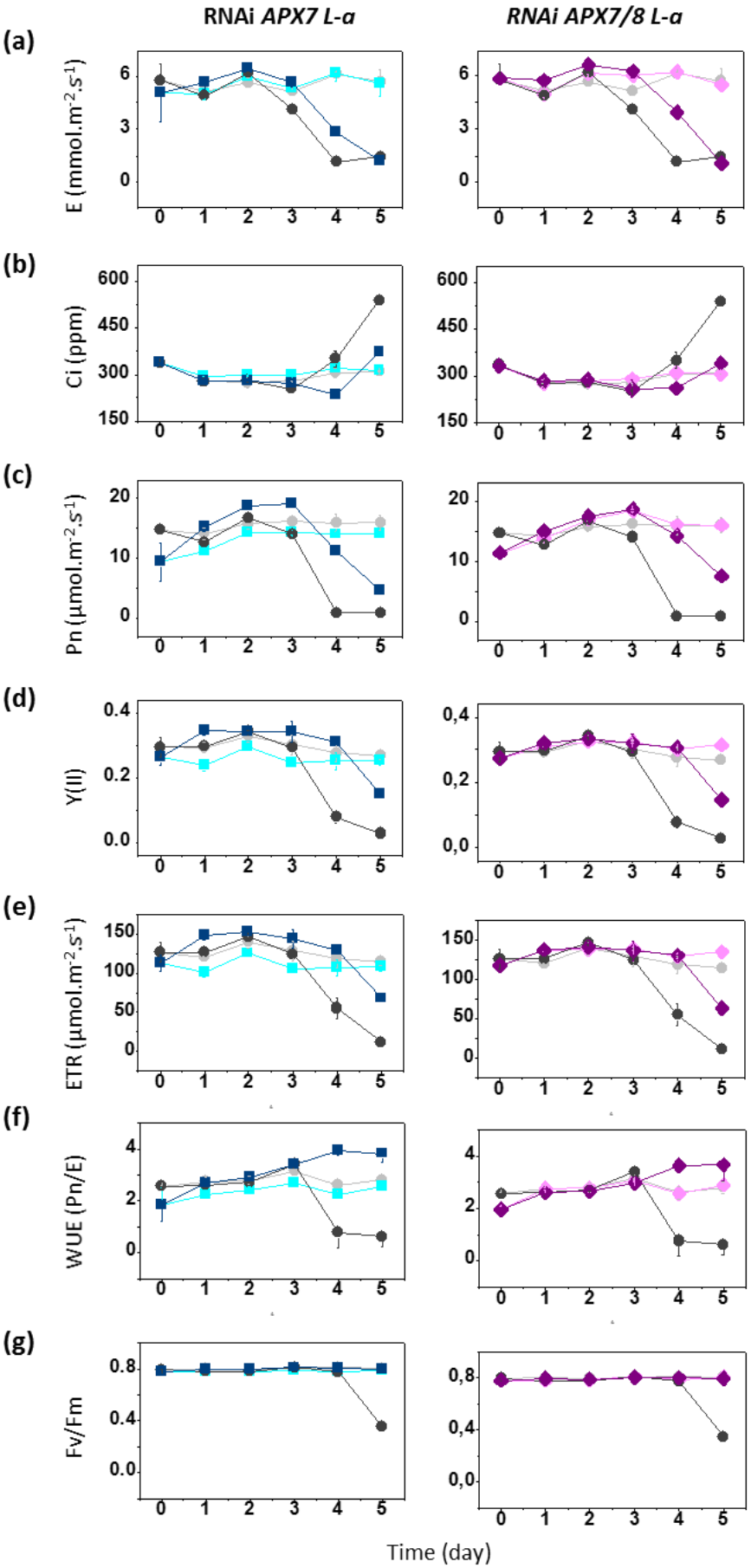

Figure 7: Measurement of photosynthetic and fluorescence parameters in plants exposed to drought stress. **(a)** transpiration, **(b)** Intracellular CO_2, **(c)** net photosynthesis, **(d)** quantum yield of photosystem II, **(e)** electron transport rate, **(f)** Water use efficiecy, **(g)** Potential (F_v/F_m) in leaves from NT (grey), RNAi*OsAPX7* (blue) and RNAi*OsAPX7/8* plants (purple). The light colors indicate the measurement in plants under control conditions. All values represent the media ± SE of at least three independent experiments.

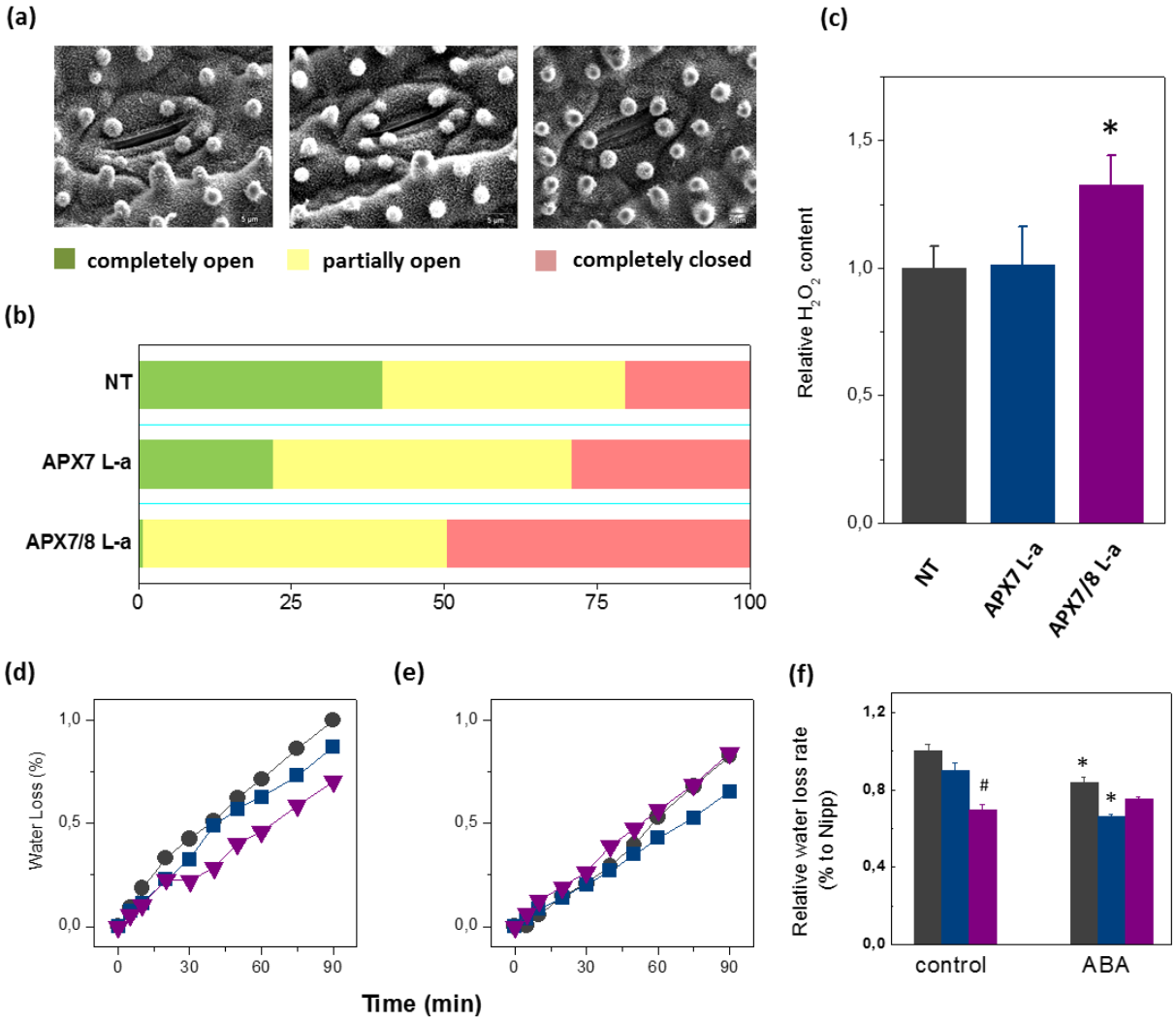
(a)
completely open
partially open
completely closed
(b)
NT
APX7 L-a
APX7/8 L-a
0
25
50
75
100
(c)
Relative H_2O_2 content
1,5
1,0
0,5
0,0
*
NT
APX7 L-a
APX7/8 L-a
(d)
Water Loss (%)
1,0
0,5
0,0
0
30
60
90
(e)
1,0
0,5
0,0
0
30
60
90
Time (min)
(f)
Relative water loss rate (% to Nipp)
1,2
0,8
0,4
0,0
#
*
*
control
ABA

Figure 8: Effect of chloroplastic APX silencing in stomata opening and in leaf desiccation. (a) Environmental scanning electron microscopy images of three levels of stomatal opening. **(b)** quantification of the percentage of the three levels of stomatal opening in leaves from NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* 30-d-old plants (n = 200 stomata for each line). **(c)** Relative content of hydrogen peroxide in leaves from NT (grey), RNAi*OsAPX7* (blue) and double RNAi*OsAPX7/8* (purple) leaves. The values represent the media ± SE of at least five independent experiments. **(d)** and **(e)** Water loss of leaves detached from plants under normal condition (treated with water) and treated with ABA 100 µM, respectively. Legend: Grey: NT, Blue: RNAi*OsAPX7* and Purple: RNAi*OsAPX7/8*. For each repeat, leaves of 30-d-old plants were used in a triplicate experiment (n = 3). The water loss rate of each conditions was quantified in the figure (f). (*) indicate populations significantly different of control situation with $p<0,05$, and (#) indicate populations significantly different of NT plants with $p<0,05$

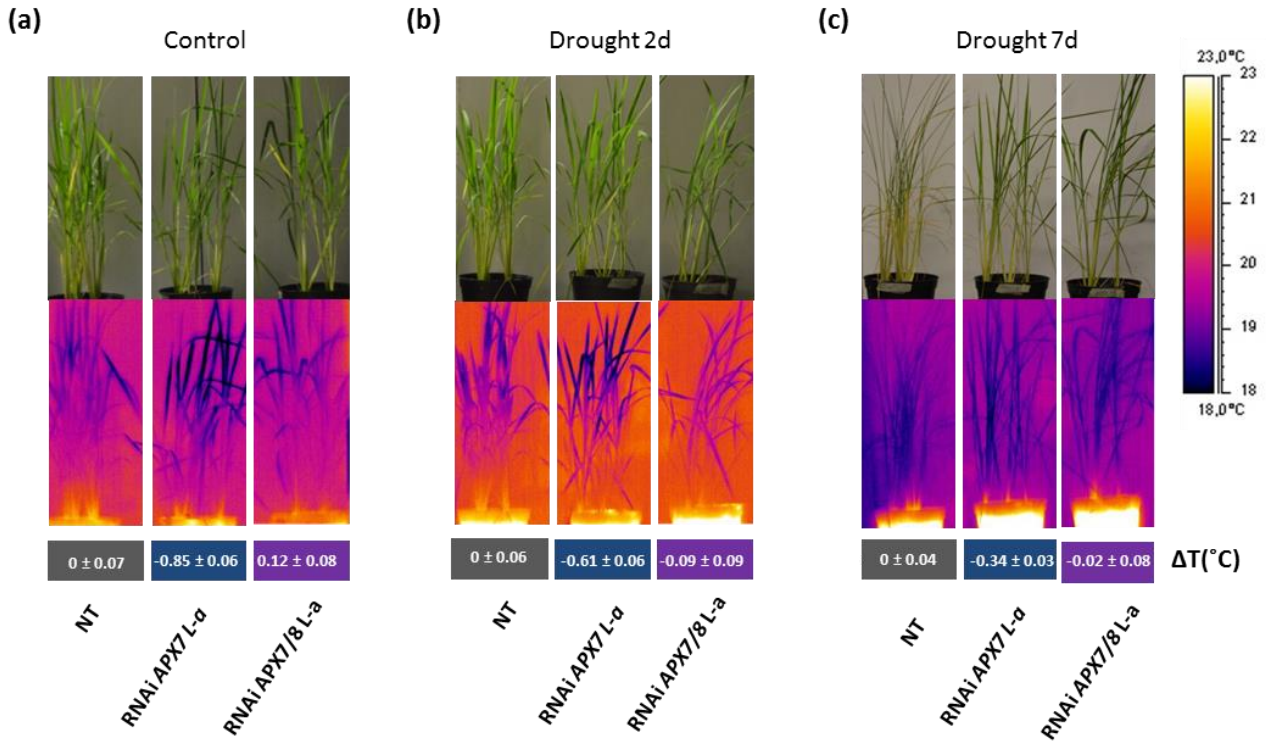

Figure 9: Thermal images of NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants captured by an infrared thermography device. (a) plants under normal condition, **(b)** plants under two days of drought stress, **(c)** plants under seven days of drought stress. The values represent the media ± SE of ΔT(˚C) of each line to NT plants.

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functionally diverse isoformslocalized in different subcellular compartments, Planta **224**: 300–314.

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SUPPLEMENTARY FIGURE

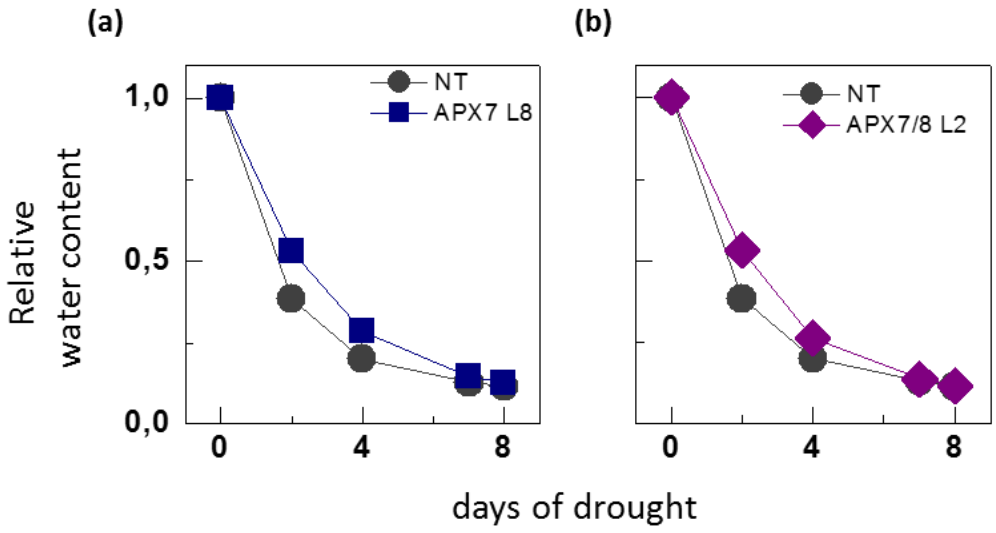

Figure S1. Relative water content in vessel of NT, RNAi*OsAPX7* and RNAi*OsAPX7/8* plants submitted to drought stress. The values represent the media ± SE of at least four independent experiments

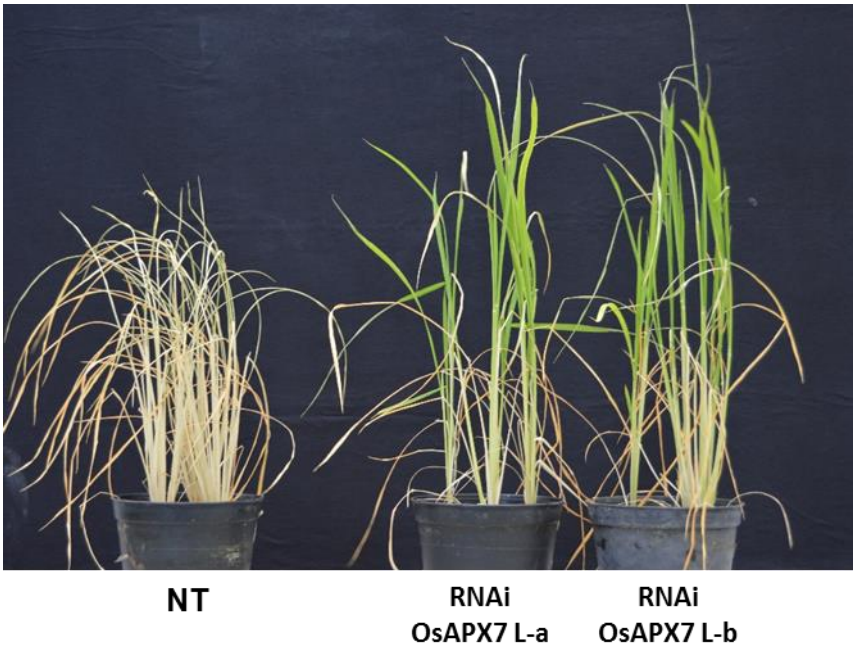

Figure S2. Effect of knockdown of chloroplastic APX in drought stress tolerance in different lines of RNAi*OsAPX7* plants. One mouth-old plants were submitted to eight day of drought and then recovered for three days.

SUPPLEMENTARY TABLE

Supplementary Table S1: Sequences of primers used in RT-qPCR experiments

Oryza sativa		
APX family		
OsAPX1	F	GGTACCTCGGAGCCGCATTTCATACCAACACA
	R	CTCGAGCTACAAGGAGGCCCACCTCA
OsAPX2	F	ACCTGAGGTCCCCTTCCA
	R	CTCTCCTTGTGGCATCTTCC
OsAPX3	F	GGAGTTGTTGCCGTTGAA
	R	ACCATCAAATCCTGATCTT
OsAPX4	F	CTCGAGTGACAAGGCATTGTTGGAAG
	R	GGTACCTCGAGCAGCTGCAGCAACAGCTACC
OsAPX5	F	CTCGAGAGGGCAATCTTGGACATCTG
	R	GGTACCTCGAGGATCAAACTTTGCCCCAAGA
OsAPX6	F	CTCGAGAGGGCAATCTTGGACATCAC
	R	GGTACCTCGAGGATCAAACTTTGCTCCGAGA
OsAPX7	F	GGTACCTCGAGGACTCGTGGTCAGGAAA
	R	CTCGAGGAGCAATCTGGGTGCAAAAT
OsAPX8	F	GGTACCTCGAGAGGAGGTCATCAGACCA
	R	CTCGAGGCTGCGAAATACTCCTACCG
Other chloroplastic peroxidases		
OsAPX-R	F	GAGAAGCCACAGCCATCTT
	R	TGCATCCTTGAAGTCGGTA
OsGPX4	F	ATGGCGTCCACCACCACC
	R	GGGCTGCCTCACCATCAGTA
Reference genes		
OsFDH3	F	TTCCAATGCATTCAAAGCTG
	R	CAAAATCAGCTGGTGCTTCTC
OsUBI	F	ACCACTTGCACCGCCACTACT
	R	ACGCCTAAGCCTGCTGGTT
OsACT2	F	GGACGTACAACTGGTATCGTGTT
	R	GTTCAGCAGTGGTAGTGAAGGAG

3.4 CAPÍTULO 4

SILENCIAMENTO OU NOCAUTE DA ASCORBATE PEROXIDASE TILACOIDAL INDUZ PADRÕES DISTINTOS DE FECHAMENTO ESTOMÁTICO E RESPOSTA AO ESTRESSE DE SECA EM ARROZ (Oryza sativa L.)

Este capítulo é referente ao manuscrito “REDUCTION OR SUPRESSION OF THYLAKOIDAL APX EXPRESSION PRODUCE DISTINCT PATTERNS OF STOMATAL CLOSURE AND RESPONSE TO DROUGHT STRESS IN RICE (*Oryza sativa L.*)” e apresenta a segunda etapa do estudo de caracterização das isoformas cloroplastídicas de ascrbato peroxidase (APX). Neste trabalho, avaliamos o papel funcional da isoforma *OsAPX8* no controle do balanço redox e do fechamento estomático em plantas de arroz. O silenciamento individual de *OsAPX8* resultou em plantas com um maior conteúdo de ROS e maior fechamento estomático em plantas crescidas em codições ideais, como foi também verificado anteriormente para as plantas duplo silenciadas para *OsAPX7* e *OsAPX8*. No entanto, as plantas silenciadas na *OsAPX8* não apresentaram uma maior tolerância ao estresse de seca. Por outro lado, o aumento da tolerância a seca também foi verificado em plantas nocaute para esse mesmo gene (apx8/apx8), as quais, curiosamente, não apresentaram as mudanças fenotípicas verificadas nas plantas silenciadas. Estes dados demonstram que o nocaute de OsAPX8 estaria gerando compensações que não ocorrem nas plantas silenciadas e que culminam no aumento da tolerância a seca.

SILENCING AND KNOCKOUT OF THYLAKOIDAL ASCORBATE PEROXIDASE LEAD TO DISTINCT PATTERNS OF STOMATAL CLOSING AND DROUGHT STRESS TOLERANCE IN RICE (*Oryza sativa L.*)

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ABSTRACT

The chloroplast ascorbate peroxidases (chlAPXs) play a central role in plant stress response. In rice plants (Oryza sativa L.) there are two chlAPXs: OsAPX7 (stromal) and OsAPX8 (thylakoidal). Previous works demonstrated that the double silencing of *OsAPX7* and *OsAPX8* genes led to plant tolerant to drought stress, with increased H_2O_2 content and stomatal closing. Curiously, the individual silencing of *OsAPX7* gene is also able to induce tolerance to drought, but without lead to differences in the biochemical or physiology parameter evaluated. In this context, the role of OsAPX8, in chloroplast redox metabolism and in drought stress respond remains unknown.

In this study we have generated rice plants individually silenced to *OsAPX8* gene (RNAi*OsAPX8*), and analyzed plants knockout to *OsAPX8* (*apx8/apx8*). Our results demonstrate that the silencing of *OsAPX8* lead to increased stomatal closing and a stress like phenotype, possibly due a lower chlAPX activity, but not induce tolerance to drought stress. Surprisingly, apx8/apx8 plants do not show biochemical and physiologic changes, but are more tolerant to drought stress. The *OsAPX8* silencing lead also a higher impact at gene expression levels, inducing mainly the expression of genes related to photosynthetic process.

In this study we characterized the role of OsAPX8 in plant development and in drought stress tolerance mechanisms. In addition, we show important differences between silencing and knockout of *OsAPX8* gene. While the silencing of OsAPX8 induce a stress-like response, the knockout of *OsAPX8* demonstrates that this gene is not essential to plant development, but in fact, its absence improve tolerance to drought stress.

INTRODUCTION

The production of reactive oxygen species (ROS) is intrinsic to aerobic metabolism, and these molecules participate of key physiological pathways, such as cell cycle, gene regulation, programmed cell death (PCD), senescence and stress response (DAT *et al*., 2000; OROZCO-CARDENAS *et al*., 2001; MULLINEAUX & KARPINSKI, 2002; VANDENABEELE *et al*., 2003). However, in high concentrations, ROS are toxic molecules, leading to oxidation of cellular structures, including DNA, proteins and membrane lipids, resulting in the disruption of metabolism and cellular structures ultimately (SCANDALIOS, 2002). Thus, plants have a range of enzymatic and non-enzymatic antioxidant mechanisms to prevent ROS from reaching toxic levels to maintain the cellular redox balance. The enzymatic mechanisms involve the ROS detoxification by action of antioxidant enzymes, as superoxide dismutases (SOD), ascorbate peroxidases (APX), catalases (CAT), glutathione peroxidases (GPx), and peroxidases (Prx) (HALLIWELL & GUTTERIDGE, 1999; SCANDALIOS, 2002; MITTLER *et al*., 2004). Among the enzymes involved in ROS metabolism, APX (EC 1.11.1.11) plays a major antioxidant role in plants, catalyzing the conversion of H_2O_2 to H_2O and O_2, using ascorbate as specific electron donor (ASADA, 1992; 1999).

In plant cell, APX is largely distributed in different subcellular compartments, as chloroplast, mitochondria, peroxisome and cytosol, where act as an antioxidant defense (ASADA, 1992). The chloroplastic APXs isoforms (chlAPX) can be composed of thylakoid-bound (tAPX) and stromal APX (sAPX), which scavenge the H_2O_2 generated mainly during photosynthesis, which is the major H_2O_2-producing metabolic process in autotrophic tissues (NAKANO & ASADA, 1981). It is estimated that the rate of accumulation of H_2O_2 in chloroplasts of higher plants under normal conditions is about 120 µm sec^{-1} (ASADA & TAKAHASHI, 1987). Thus, the immediate scavenging of H_2O_2 is indispensable to maintain the photosynthetic activity of chloroplasts.

Due to the importance of APX activity in H_2O_2 metabolism, it has been proposed that APX play a central role in plant stress response. In fact, Cotton plants (*Gossypium hirsutum*) that overexpress recombinant APX in chloroplasts showed an enhanced resistance to chilling-associated oxidative stress (PAYTON *et al*., 2001), and a similar result was found in tobacco (*Nicotiana toabacum*), where the overexpression of tAPX led to more resistant to chilling and methyl viologen (MV) treatment (YABUTA *et al*., 2002). In wheat (*Triticum aestivum* L.), knockout to tAPX led to reduced photosynthetic activity and growth (DANNA *et al*., 2003). In previous work, our group demonstrated that in rice (*Oryza sativa* L.) the chAPX (*OsAPX7* and *OsAPX8*) have a distinct pattern of expression in response to different abiotic stresses, as drought, high light and treatments with H_2O_2 or MV, being *OsAPX7* induced and *OsAPX8* repressed (TEIXEIRA et al, 2006; ROSA et al, 2010). In addition, we demonstrated that the double silencing of *OsAPX7* and *OsAPX8* do not led to phenotype differences in rice plants under optimal condition of growth, but led to differences in photosynthetic parameters related to efficiency of light utilization and CO_2 in stress conditions (CAVERZAN *et al*., 2014). Note that the opposite expression of *OsAPX7* and *OsAPX8* in response to stress could hamper the interpretation of double silencing, and justify the absence of phenotype differences in plants under normal condition. For all this, it would be important perform the individual knockout or knockdown of each chAPX to ensure the study of the physiological role of each gene. In fact, recently we demonstrated that the silencing of OsAPX7 in rice plants improve an increased tolerance to drought stress, without modify the stomata opening (JARDIM-MESSEDER et al, not published yet).

Here, we performed a functional study of thylakoidal ascorbate peroxidase (*OsAPX8*) gene, through rice plants individually silenced to *OsAPX8* (RNAi*OsAPX8*) or knockout mutant at *OsAPX8* gene (*apx8/apx8*). Our results demonstrate that RNAi*OsAPX8* plants show a decrease of growth rate, and a high hydrogen peroxide content, even in normal condition. Furthermore, the silencing of OsAPX8 led to decrease in stomatal opening and increase in leaves surface temperature. In contrast, this phenotype has not been verified in apx8/apx8 plants, which are similar to nom-transformed (NT) plants in normal

condition. However, beside the silencing of *OsAPX8* lead to a stress-like phenotype, only *apx8/apx8* plants show an increased tolerance to drought stress. These results were also confirmed by physiological parameters, as gas exchange and chlorophyll fluorescence. In addition, RNAseq analysis confirmed that, in normal conditions, the silencing of *OsAPX8* led to a higher impact on plant than the knockout, regulating mainly the expression of genes related to photosynthesis and signal transduction,

MATERIAL AND METHODS

Plant material and growth conditions

Rice (*Oryza sativa* L. japonica cv. Nipponbare) seeds were germinated in MS medium (Sigma-Aldrich) at 25°C with a 12-h photoperiod. One week after being sown, the rice seedlings were transferred for hydroponic in 200-ml plastic cups (three seedlings per cup) filled with Hoagland–Arnon's nutritive solution (HOAGLAND & ARNON,1950). To ABA treatment, 100µM ABA was dissolved in 0.1% Triton-X-100 and sprayed in excess on the leaves of the one-month-plants until complete wetting was achieved.

Methyl viologen (MV) treatment

The one-month-old plants were used to MV treatment. These plants were grown as described previously. 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.

Cold stress

The plants of one-month old were used to cold treatment. The plants were grown as described previously and submitted to 10°C temperature. Leaves were collected at 6 and 24 hs after the start of cold treatment.

Drought stress

To drought experiments, the one-week-old rice seedlings were transferred to soil in plastic vessels. Was used a mixture of soil, vermiculite and peat (2:1:1) and was placed six plants per vessel. The drought experiment was performed in one-month-old plants, thought the suspense of watering. The drought stress was maintained until the relative water content in vessel reaches 10% (in about eight days). The control of relative water content during drought stress experiment is showed in Supplementary figure S1.

Vector construction and plant transformation

A chimeric gene producing mRNA with a hairpin structure (hpRNA) was constructed based on the sequence of the *OsAPX8* (LOC_Os02g34810) gene. The following primer pairs were used to amplify a 227 bp RNAi*OsAPX8* sequence: 5'- CTCGAGGCTGCGAAATACTCCTACGG-3' and 5'-GGTACCTCGAGAGGAGGTCATCAGACCATCG-3'. PCR products were cloned into the Gateway vector pANDA, which hairpin RNA is driven by the maize ubiquitin promoter and an intron placed upstream of the inverted repeats (MIKI & SHIMAMOTO, 2004). The construct was denominated RNAi*OsAPX8.* Agrobacterium tumefaciens-mediated transformation was performed as described previously (UPADHYAYA *et al*., 2002). The *apx8/apx8* knockout plants were obtained by TOS-17 retrotransposson insertion in exon 4 (Supplementary fig. S1A). All plants used in this work are homozygous as demonstrated in Supplementary figure S1.

Quantitative real-time PCR (RT-qPCR)

Real-time PCR experiments were carried out using cDNA synthesized from total RNA purified with TRIzol (Invitrogen®). Complementary (c) DNA was obtained using the SuperscriptTMII (Life Technologies®) reverse transcriptase system and a 24-polyTV primer (Invitrogen®). After synthesis, cDNAs were diluted 10–100 times in sterile water for use in PCR reactions. All reactions were

repeated four times, and expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method (LIVAK & SCHMITTGEN, 2001; SCHMITTGEN & LIVAK, 2008). RT-qPCR reactions were performed in an Applied Biosystems StepOne plus Real Time PCR system (Applied Biosystems®) using SYBR-green intercalating dye for fluorescence detection. The primer sequences and reference genes are listed in Supporting Information Table S1. The data from the experiments relative to the plants submitted to H_2O_2 treatment and drought were obtained from ROSA *et al*. (2010), while experiments relative to MV and high light were obtained from CAVERZAN et al, 2014.

Diaminobenzidine (DAB) staining and quantitative measurement of H_2O_2

The hydrogen peroxide was detected by DAB staining as described previously (THORDALCHRISTENSEN *et al*., 1997). The fully expanded leaves of 20 days-old plants were detached and incubated overnight in DAB staining solution (1 mg ml^{-1} DAB in 10 mM MES, pH 6.5) at 28 °C and cleared in boiling propanone for 10 min before photographing.

Measurements of H_2O_2 production was performed by extracting H_2O_2 from leaves according to RAO *et al*. (2000) using the Ampliflu Red (Sigma-Aldrich) oxidation (SMITH *et al*., 2004). Fluorescence was monitored using a fluorometer at excitation and emission wavelengths of 563 nm (slit 5 nm) and 587 nm (slit 5 nm), respectively. Calibration was performed by the addition of known quantities of H_2O_2.

Pigment determination

The total chlorophyll, chlorophyll a and b were determined after extraction in ethanol spectrophotometrically at 663 and 649 nm. The amount of pigment was calculated using the equations proposed by LICHTENTHALER & WELLBURN (1983):

$$\text{Chlorophyl a (mg/L)} = 12{,}25 \times Abs_{663} - 2{,}79 \times Abs_{647}$$

$$\text{Chlorophyl b (mg/L)} = 21{,}50 \times Abs_{647} - 5{,}10 \times Abs_{663}$$

Membrane damage determination

Electrolyte leakage (membrane damage) was measured as described by BLUM & EBERCON (1981). Leaf slices were placed in tubes containing 10mL of deionized water. The flasks were incubated in a shaker for 12 h, and the electric conductivity in the medium (L1) was measured. Then, the medium was boiled (95 °C) for 60 min and the electric conductivity (L2) was measured again. The relative membrane damage (MD) was estimated by MD = L1/L2×100.

Imaging of rice stomata

The stomata were visualized by scanning electron microscopy and subsequently quantified by counting class. Leaves of 30 day-old plants were detached and the samples are prepared as HUANG *et al*., (2009).

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. The following PSII parameters were measured: $\Delta F/F'_m$, 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 = ($\Delta F/F'm \times PPFD \times 0.5 \times 0.84$), 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 [$qP = (F'_m- F_s)/(F'_m-F'_o)$] and the non-photochemical quenching coefficient [$NPQ =(F_m- F'_m)/F'_m$], where F_m and F_o are, respectively, maximum and minimum fluorescence of dark-adapted leaves; F'm and F_s are, respectively, maximum and steady state fluorescence in the light-adapted state, and F'_o 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 ºC and relative humidity of 78% and vapor pressure deficit (VPD) of 1.8 Pa. The gas exchange variables measured were net photosynthesis (PN) and intercellular CO_2 concentration (CI).

RNAseq analysis

To RNAseq analysis, total RNA was purified from one month old plants, using TRIzol (Invitrogen®). Was evaluated two simples for lines (NT, RNAi*OsAPX8*, *apx8/apx8*), compoused by mix of total RNA from five plants. The plant growth condirions was the same showed above. The RNAseq was made by Centro de Genômica Funcional - USP (Piracicaba, Brasil). In the results is show the average of the relative values of expression for each line compared to NT plants. Was considered differently expressed the values with $p<0,03$.

Gene ontology analysis

Gene ontology analyses were performed by the web-based tool and database agriGO (DU *et al*., 2010).

Functional protein association networks

The functional protein association network was created using the STRING database (SNEL *et al*., 2000) and analyzed by Medusa (HOOPER & BORK, 2005) and ViaComplex software (CASTRO *et al*., 2009)**.**

Protein determination

Protein concentrations were determined as described by BRADFORD (1976) using BSA as a standard.

Statistical analysis

Data were plotted with GRAPHPAD PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA) and analyzed by one-way ANOVA and a posteriori Tukey's test. P-values of 0.05 were considered statistically significant.

RESULTS

OsAPX8 expression is down-regulated in response to abiotic stress

In a previous work, we demonstrated that the expression of *OsAPX7* and *OsAPX8* is modulated in an antagonic way in the response to abiotic stress, being *OsAPX7* generally induced, while *OsAPX8* transcript level is generally down-regulated (TEIXEIRA *et al*., 2006; ROSA *et al*., 2010; CAVERZAN *et al*., 2014). Due the importance of H_2O_2 metabolism in stress response, we compared the expression profile of *APX* family genes in rice plants exposed to different abiotic stress, as drought, H_2O_2 treatment, MV treatment and high light, descripted by Rosa *et al*. (2010) and CAVERZAN *et al.* (2014). Furthermore, we added in this analysis the expression profile of APX family in rice plants exposed to cold stress (Fig. 1). These results confirmed the antagonic expression pattern of *OsAPX7* and *OsAPX8* genes in response to abiotic stress.

Molecular and biochemical analyses of RNAiOsAPX8 and apx8/apx8 plants

To determine the functional role of the thylakoidal APX and the mean of the reduction of its expression in response to abiotic stress, the *OsAPX8* gene was silenced using inverse repeat (IR) constructs that direct transcription of dsRNA (hairpin). Rice calli were transformed via *Agrobacterium* with a RNAi*OsAPX8* construct, and transgenic lines carrying the hairpin construct were recovered. In addition, in this work, we include in the analysis a mutant plant knockout to *OsAPX8* gene (*apx8/apx8*) by TOS17 retrotransposon insertion.

The RT-qPCR analysis of five lines of RNAi*OsAPX8* T2 plants showed that the *OsAPX8* mRNA levels were reduced around 90%, while in mutant plants it was not detected (Fig. 2H). In addition, the silencing of *OsAPX8* induce the up regulation of other OsAPX genes, as *OsAPX3* (Fig. 2C), *OsAPX4* (Fig. 2D), *OsAPX5* (Fig. 2E) and *OsAPX7* (Fig. 2G). In addition, our results demonstrated

that the expression of other peroxidases located in chloroplast, as *APX-related* (*APX-R*) and *OsGPX4* were also induced in RNAi*OsAPX8* plants (Fig. 2I and 2J, respectively). On the other hand, the knockout of the OsAPX8 gene (*apx8/apx8* plants) induced the expression of *OsAPX4*, *OsAPX7*, *OsAPX-R* and *OsGPX4*, but not *OsAPX3* and *OsAPX5*. These results suggest that the silencing of *OsAPX8* by RNAi induced a higher impact in the expression of other peroxidases. In addition, *OsAPX1*, *OsAPX2* or *OsAPX6* were not changed neither in RNAi*OsAPX8* or *apx8/apx8* plants (Fig. 2A, 2B and 2F, respectively), except only one line of RNAi*OsAPX8* plants which showed decrease in *OsAPX2* expression (Fig. 2B). In the further experiments the T2 generation of the RNAi*OsAPX8* line-d was used as the representative line.

To verify the effect of OsAPX8 silencing or knockout in total chloroplastic APX activity, we determine the APX activity in isolated chloroplast using different H_2O_2 concentrations. Our results demonstrate that RNAi*OsAPX8* plants show a lower chloroplastic APX activity than NT plants (Fig. 3A). On the other hand, the knockout of OsAPX8 do not lead to changes in chloroplastic APX activity (Fig. 3B), indicating a possible compensatory mechanism that maintain the chloroplastic APX activity in *apx8/apx8* plants, but is not present in RNAiOsAPX8 plants. In addition, we determine the catalytic parameter of chloroplastic APX activity. In fact, RNAiOsAPX8 plants show a lower V_{MAX} of chloroplastic APX activity (Fig. 3C) and a higher K_M to H_2O2 (Fig. 3D), resulting a lower catalytic efficiency (Fig. 3E). As expected, apx8/apx8 plant do not show difference in catalytic parameters of chloroplastic APX activity.

The silencing and the knockout of *OsAPX8* lead to differences in plant growth and in response to chloroplastic stress induced by MV treatment

To verify the effect of *OsAPX8* silencing or knockout in plant development, the growth of RNAi*OsAPX8* and *apx8/apx8* plants was analyzed. Under normal conditions, the transgenic plants presented a delayed growth when compared to the NT plants grown under normal conditions. However, *apx8/apx8*

mutant plants did not present an apparent altered phenotypic, showing the same appearance than NT plants (Fig. 4).

Due the importance of APX activity in chloroplast redox homeostasis, we verify the effect of silencing and knockout of *OsAPX8* gene in chloroplast stress response. We evaluated the change of chlorophyll content in leaves of plants treated with MV, which is able to induce ROS production in the chloroplast. Our results show that RNAi*OsAPX8* and *apx8/apx8* plants showed a higher decreasing of chlorophyll content after MV treatment compared to the NT plants (Fig. 5A-5C), indicating that these plants are more susceptible to MV treatment than NT plants. It is important to note that under normal conditions the RNAi*OsAPX8* plants show naturally a lower chlorophyll content, while no difference was verified in apx8/apx8 plants. In spite of that, the treatment affects more the plants deficient in OsAPX8, revealing that this gene is essential for the protection of the chloroplast.

OsAPX8 knockout led to an improved drought tolerance in rice plants

Previous works have demonstrated that the drought tolerance in transgenic plants and tolerant cultivars is highly dependent of ROS production control and scavenging in chloroplast (VAN CAMP *et al.*, 1996; HERNANDEZ *et al.*, 2001; MITTLER & BERKOWITZ, 2001; TSENG *et al.*, 2007). Thus, we evaluated the effect of the silencing and knockout of *OsAPX8* gene in rice drought tolerance. Our results demonstrated that the knockout plants showed higher drought tolerance when compared to NT and RNAi*OsAPX8* plants (Fig. 6A and 6B). We also measured the cell membrane damage in leaves of plants submitted to drought stress. Under drought stress, *apx8/apx8* plants present lower cell membrane damage (Fig. 6C). On the other hand, RNAi*OsAPX8* plants presented the cell membrane damage similar to NT plants. (Fig. 6C). These results indicated that the knockout of *OsAPX8* gene significantly improves drought tolerance in rice plants.

To elucidate the physiological mechanism of drought tolerance in mutant plants, we compared gas exchange and photochemical parameters

between NT, RNAi*OsAPX8* and *apx8/apx8* plants under drought stress condition. NT and RNAi*OsAPX8* plants showed a decrease of apparent transpiration (E), net photosynthesis (Pn), photosystem II activity (Y) and electron transport rate (ETR) in the fourth day of drought condition, while *apx8/apx8* plants maintain these parameters, being affected by drought stress only at the fifth day (Fig. 7A, 7C, 7D, 7E, respectively). Consequently, the *apx8/apx8* plants show an increase of internal CO_2 concentration later than NT or RNAi*OsAPX8* plants (Fig. 7B). In addition, the higher drought tolerance of *apx8/apx8* plants is confirmed by the increased ability of these plants to maintain the water use efficiency (WUS) and the potential quantum yield of photosystem II (Fv/Fm) under drought stress condition (Fig. 7F and 7G, respectively).

The *OsAPX8* silencing, but not knockout, causes decrease of stomatal opening

Due the importance of stomata in the responses to drought (HETHERINGTON & WOODWARD, 2003), we have also examined the stomata status in NT, RNAi*OsAPX8* silenced and *apx8/apx8* knockout plants (Fig. 8A). Our results showed that about 50% of stomata were completely closed in the RNAi*OsAPX8* silenced plants, while only 20% of stomata were completely closed in NT and *apx8/apx8* plants. In addition, NT and *apx8/apx8* plants showed 39.8% and 24% of stomata completely opened, respectively, while in RNAi*OsAPX8* plants only 5.5% of stomata were completely opened. The percentage of partially open stomata was 39.8%, 45.1% and 53.5% in NT, RNAi*OsAPX8* silenced and *apx8/apx8* plants, respectively (Fig. 8B). These results showed that *OsAPX8* silencing led to a significant decrease of stomatal opening, indicating that stomata movement is greatly affected by *OsAPX8* silencing in rice plants, but not by knockout of this gene.

Previous work has demonstrated that ROS is highly involved in stomata closure, mainly in response to drought stress (DASZKOWSKA-GOLEC & SZAREJKO, 2013). The measurement of the H_2O_2 content in leaves of all plants

revealed that, in fact, RNAi*OsAPX8* plants have an increase of 60% in H_2O_2 level, while no differences were observed between NT and *apx8/apx8* plants (Fig. 8C).

To verify the effect of *OsAPX8* silencing and knockout in leaf water loss, we submitted rice leaves to desiccation experiment. The *OsAPX8* silencing led to a lower leaf desiccation (25%), while leaves from *apx8/apx8* and NT plants showed the same water loss rate. Interestingly, the pre-treatment with abscisic acid (ABA), which induce stomatal closure, led to a decrease of leaf desiccation in NT and *apx8/apx8* plants, but do not change the leaf desiccation in RNAi*OsAPX8* plants. Under this condition, there are not differences in leaf desiccation between NT, RNAi*OsAPX8* and *apx8/apx8* plants (Fig. 8E-8G) indicating that the lower leaf desiccation in RNAi*OsAPX8* plants is possibly due a higher stomatal closure.

Other alternative approach to measure stomatal conductance is the infrared (IR) thermography. The infrared measurements of leaf temperature correlate well with estimates of stomatal conductance (JONES, 1999; SIRAULT *et al*., 2009) and consequently in rice water status (CAO *et al*., 2013). Thus, we measured the leave surface temperature in NT, RNAi*OsAPX8* and *apx8/apx8* plants under normal condition, moderate drought stress (2 days) and severe drought stress (7 days). Our results demonstrate that in normal condition, the RNAi*OsAPX8* plants showed naturally an increase of about 0.48 ºC in leaf surface temperature (Fig. 9A). However, under moderate and severe drought stress no difference in leaf surface temperature between all plants was observed (Fig. 9B and 9C). These results indicate that only the silencing of *OsAPX8* gene led to changes in leaf surface temperature in normal condition, and interestingly, this effect was not induced by the knockout of *OsAPX8*.

RNAseq analysis of RNAi*OsAPX8* and *apx8/apx8* plants

RNAseq analyses were performed to identify differentially expressed genes in RNAi*OsAPX8* and *apx8/apx8* plants with one-month old grown under normal conditions. Our results demonstrate differential expression between RNAi*OsAPX8* and NT plants, as well as, *apx8/apx8* and NT plants

(Supplementary Table S2). In RNAi*OsAPX8* plants 222 genes were significantly upregulated when compared to NT plants ($p<0.03$), while only 17 genes were significantly upregulated in *apx8/apx8* plants when compared with NT plants (Fig. 10A). On the other hand, 121 genes were down-regulated in RNAi*OsAPX8* plants, while only 62 genes were down-regulated in *apx8/apx8* plants (Fig. 10B). A set of proteins which showed significantly difference in expression when compared to NT plants were also analyzed and classified into groups according to their ontology (GO: metabolic process). Ontological analysis showed that in RNAi*OsAPX8* plants, among the up-regulated genes, those related to photosynthesis was well represented (14.8% of the total genes up-regulated). In addition, there is an increase of genes related to light reaction and harvesting (7.1% and 6.5%, respectively), generation of energy (8.2%) and genes related to metabolism of organic molecules. On the other hand, among the down-regulated genes, the most representative were those related to protein metabolic process (25.6%) and to signal propagation function, such as genes related to protein phosphorylation (20.5%) and post-translation modification (20.5%) (Fig. 10C). The representability of these ontologies among the genes up and down-regulated is higher than their representability in rice genome, indicating that these ontological groups are significantly modulated by *OsAPX8* silencing. In KO plants, there is not an enrichment of a specific ontological group in up-regulated genes. In addition, to down-regulated genes, those related to metabolic process, primary metabolism and cellular process were well represented (34.4%, 26.5% and 20.3%, respectively) (Fig. 10D). However, the representability of all ontologies in the input of analyzed genes is lower than their representability in rice genome. These data indicate that the knockout of *OsAPX8* do not led to modulation of a specific ontological group.

Due the higher representability of photosynthesis related genes in up-regulated genes, the transcription profile of an association network of photosynthesis related genes (GO:0015979) was analyzed comparing NT, RNAi*OsAPX8* and *apx8/apx8* plants. This network was created using the STRING database (SNEL *et al*., 2000) with genes related to photosynthesis in rice. Our results demonstrate that the silencing of *OsAPX8* led to differential

expression of the genes belonging to this network, while in *apx8/apx8* plants it was not verified similar effect (Fig. 11A). In addition, the relative expression of genes with ontology “photosynthesis”, was quantified. In RNAi*OsAPX8* plants, there are significantly groups of up and down-regulated genes, while in *apx8/apx8* plants, in general there are not differential expression of these genes (Fig. 11B). Finally, these results demonstrate that, the silencing of *OsAPX8* also led to a higher impact in gene expression than the complete *OsAPX8* knockout.

DISCUSSION

Many studies have demonstrated a close relationship between redox signals and acclimation to abiotic stresses. The chloroplast plays a central role in redox/ROS-mediated systemic signaling and acclimation responses (KARPINSKI *et al.,* 1999; MULLINEAUX *et al*., 2006; MÜHLENBOCK et al., 2008; SZECHYŃSKA-HEBDA *et al*., 2010). The overexpression or knockout of chlAPX led to an altered response to abiotic stress in different species, as cotton (PAYTON *et al*., 2001), tobacco (YABUTA *et al*., 2002) and wheat (DANNA *et al*., 2003). In rice, there are two exclusive isoforms of chlAPX, and we have previously demonstrated that abiotic stress, as high light or MV, led to contrasting response related to *OsAPX7* and *OsAPX8* expression: *OsAPX7* expression is up-regulated, while *OsAPX8* expression is downregulated (CAVERZAN *et al*., 2014). Similarly, H_2O_2 treatment and drought also induced *OsAPX7* expression and decreased *OsAPX8* expression (ROSA *et al*., 2010). Here, we have confirmed that this antagonism also occurred in response to other abiotic stress, as cold. Due to the general and conserved decrease of *OsAPX8* expression in plants submitted to different abiotic stress we hypothesized that the down modulation of *OsAPX8* expression play an important role in rice stress response.

To understand the physiologic role of *OsAPX8*, we performed the molecular, physiological and biochemical characterization of the effect of silencing and knockout of *OsAPX8* gene in rice plants. Our results demonstrated that the silencing of *OsAPX8* led to a compensatory expression of other APX isoforms, as *OsAPX3*, *OsAPX4*, *OsAPX5* and *OsAPX7*, and of other chloroplastic peroxidases, as *OsAPX-R* and *OsGPX4*. In addiyion these plants show a lower chloroplastic APX activity, accompanied by a a higher H_2O_2 accumulation, and a delayed growth, compared to NT plants. As described by DASZKOWSKA-GOLEC & SZAREJKO (2013) the increase of ROS production is also involved with stomata closure mechanism, and in fact, the RNAi*OsAPX8* plants have a lower number of opened stomata, which would limit the CO_2 assimilation to plant growth. In addition, the RNAi*OsAPX8* plants showed an increased leaf surface

temperature, even in normal condition, which confirm the lower stomatal conductance. Together, these results suggest that the *OsAPX8* silencing induce a stress-like response in rice plant, simulating the natural response of *OsAPX8* expression in plants submitted to stress. On the other hand, the complete knockout of *OsAPX8* did not produce any apparent phenotype alteration, and *apx8/apx8*plants maintained a normal growth and development, and a similar H_2O_2 content, stomatal conductance and leaf surface temperature compared to NT plants. However, both silencing and knockout plants show a lower tolerance to MV treatment, demonstrating that *OsAPX8* expression is fundamental to chloroplast stress response, possible by its H_2O_2 scavenger activity

Previous studies have demonstrated that ROS production control and scavenging by chloroplast play an important role, not only in stomatal conductance, but also in plant tolerance to drought (VAN CAMP *et al*., 1996; HERNANDEZ *et al*., 2001; MITTLER & BERKOWITZ, 2001; TSENG et al, 2007). In fact, previously, we demonstrated that the silencing of OsAPX7, another chlAPX, led to physiological changes, as increased transpiration, and improve drought tolerance in rice plants (Jardim-Messeder et al, submitted work). Thus, we submitted the RNAi*OsAPX8* and *apx8/apx8* plants to drought stress, to also determine the role of *OsAPX8* in drought stress response. Surprisingly, the *apx8/apx8* plants, but not the RNAiOsAPX8, showed a higher drought tolerance. This result was confirmed by a lower membrane damage and by the evaluation of photochemical parameters and gas exchange in plants exposed to drought stress. Our results revealed that *apx8/apx8* plants are able to maintain the transpiration, and consequently stomatal conductance, for a longer time under drought stress. The maintenance of stomatal conductance under stress condition allowed the plant to keep the photosynthesis activity, resulting in an increasing of the intracellular CO_2 concentration. The higher stomatal conductance of *apx8/apx8* plants possibly enables the constant offers of CO_2 to RUBISCO carboxylase activity, leading the ribulose bisphosphate (RuBP) to Calvin cycle and preventing the photorespiration. Therefore, to promote the re-oxidation of NADPH to NADP, the maintenance of the Calvin cycle under stress condition enables the continuous electron transporter chain activity, impairing the

photosynthesis inhibition. Consequently, under drought stress condition, the *apx8/apx8* plants are able to maintain for a longer time the water use efficiency and the potential quantum yield of photosystem II.

The comparative analysis of the transcriptome of RNAi*OsAPX8* and *apx8/apx8* plants revealed that the silencing of *OsAPX8* caused higher impact in the global gene expression than the complete gene knockout did. The ontological analysis of differently expressed genes demonstrated that photosynthesis related genes are the most well represented ontological group, while the main down-regulated genes corresponded to protein signaling, as post-transductional modification, phosphorylation, and to phosphate and phosphorus metabolism. In addition, among the down-regulated genes there are an increased representation of genes related to ontologies “photosynthesis” “photosynthetic electron transport chain”, “light reaction” and “photosynthetic electron transport in photosystem II”, though these groups were lower represented than other ontology being indicated as “other”. On the other hand, the knockout of *OsAPX8* did not led to a significant ontological difference. These data confirmed that the RNAi*OsAPX8* plants also showed a higher difference at gene expression level, confirming our hypothesis that the *OsAPX8* silencing led to a stressed-like phenotype in rice plants. In fact, previous works demonstrated that drought stress significantly affect photosynthesis related gene expression in rice (HAZEN *et al.*, 2005; WANG *et al.*, 2011) and this group of genes is highly affected by the *OsAPX8* silencing, but not by the complete knockout of *OsAPX8*.

In this work we characterized the functional role of *OsAPX8* in plant development and demonstrated the importance of *OsAPX8* in drought stress response. While the silencing of *OsAPX8* simulate a natural response to stress, leading to a stressed-like phenotype, the knockout of *OsAPX8* should trigger an unknown mechanism able to maintain a normal phenotype, and still confer tolerance to drought stress. This mechanism could involve the genes differently expressed in *apx8/apx8* plants, as for example LOC_Os08g27170.1, which encode a calmodulin binding protein and is overexpressed in these plants. Previous work, demonstrated in *Arabidopsis thaliana*, that the overexpression of homologue protein CBP60g is a positive regulator of both disease resistance and

drought tolerance. Further studies should be conducted to determine the exact mechanism by which *OsAPX8* knockout confers drought tolerance in rice plants.

FIGURES

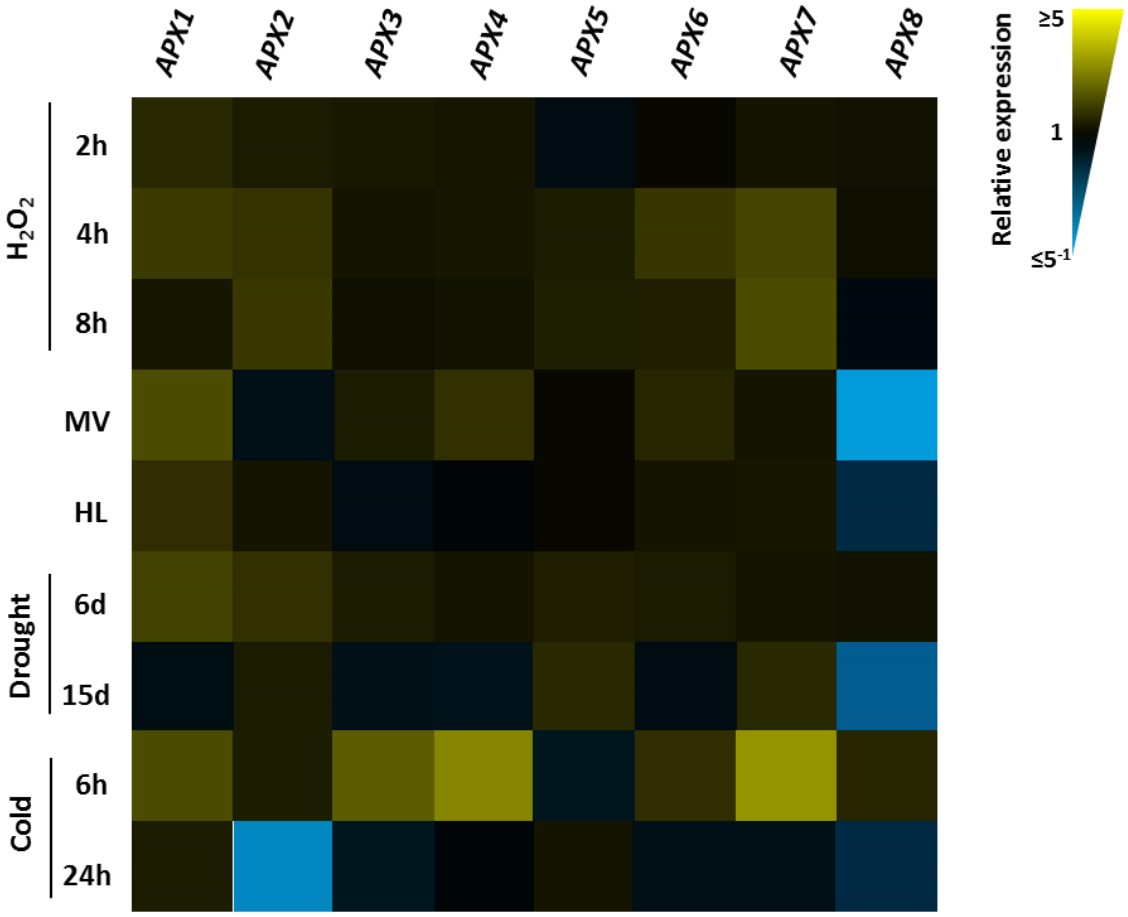


Figure 1. Relative expression of APX family in rice leaves from plants submitted in to different stress conditions. *The values were normalized by the at least four constitutive genes and represent the media ± SE of at least three independent experiments.*

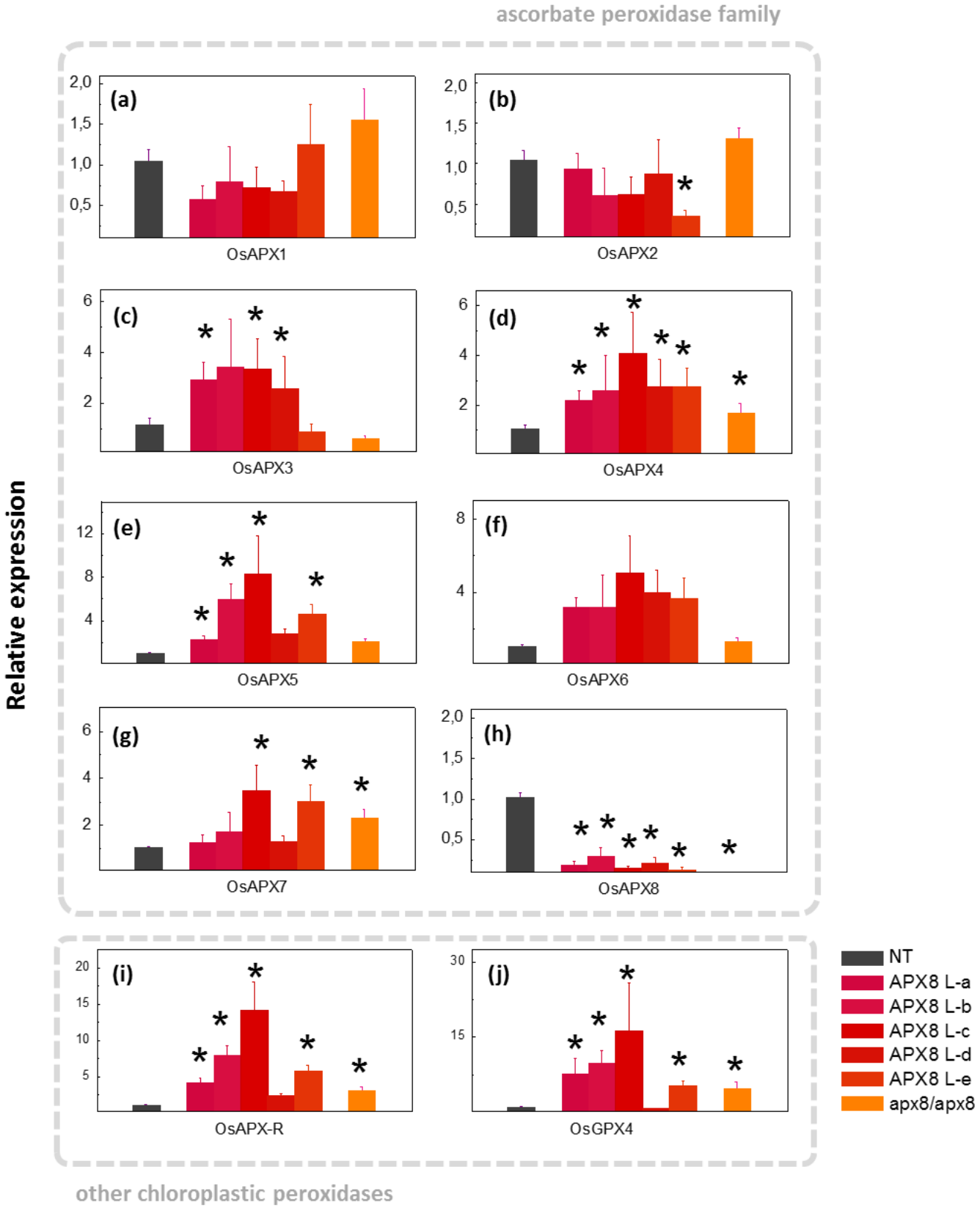

ascorbate peroxidase family
Relative expression
(a)
OsAPX1
(b)
OsAPX2
(c)
OsAPX3
(d)
OsAPX4
(e)
OsAPX5
(f)
OsAPX6
(g)
OsAPX7
(h)
OsAPX8
(i)
OsAPX-R
(j)
OsGPX4
other chloroplastic peroxidases
NT
APX8 L-a
APX8 L-b
APX8 L-c
APX8 L-d
APX8 L-e
apx8/apx8

Figure 2. Quantitative determination of ascorbate peroxidase family and other chloroplastic peroxidases mRNA in leaves of NT (non-transformed), RNAiOsAPX8 and apx8/apx8 plants. *The values were normalized by the at least three constitutive genes and represent the media ± SE of at least three independent experiments. (*) indicate populations significantly different with p<0,05.*

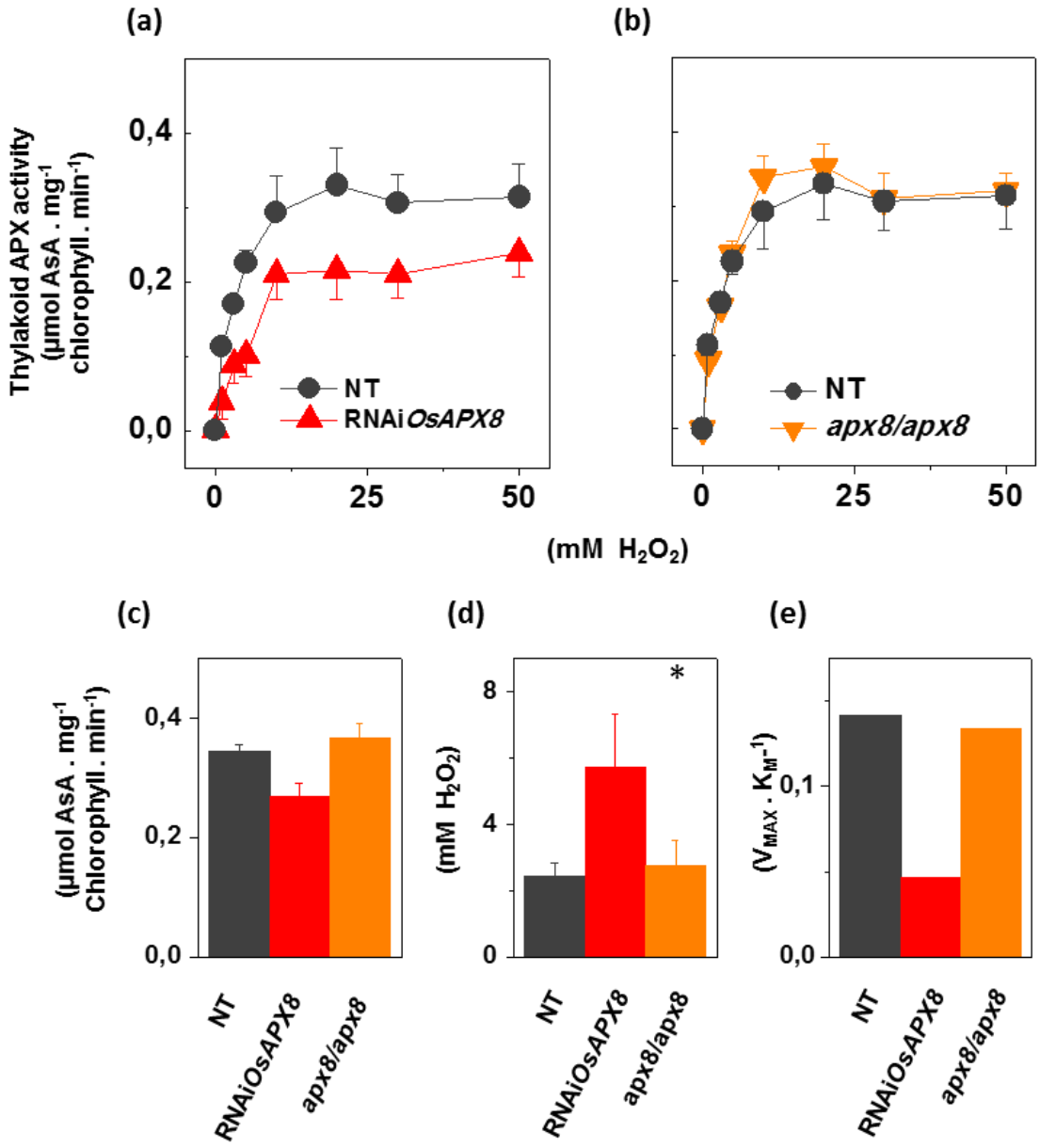
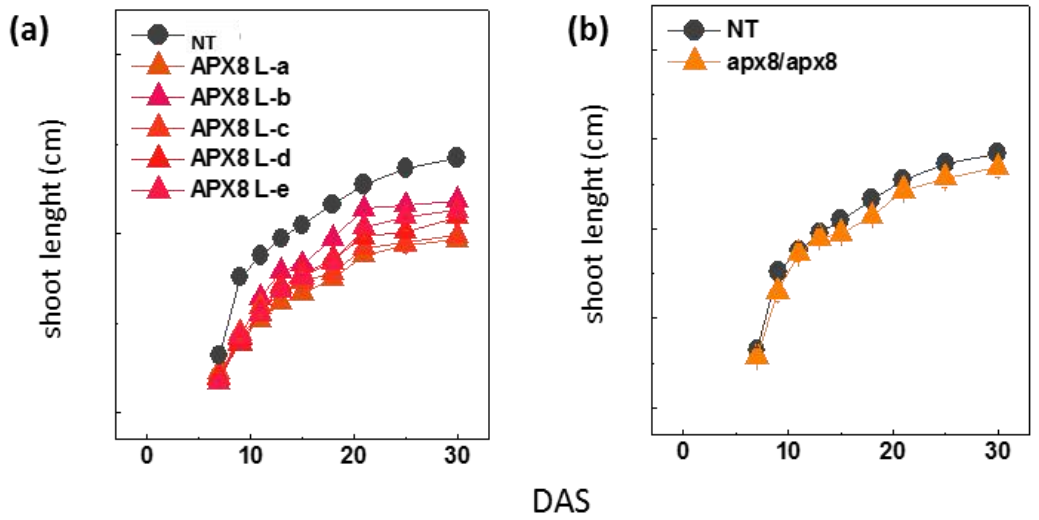
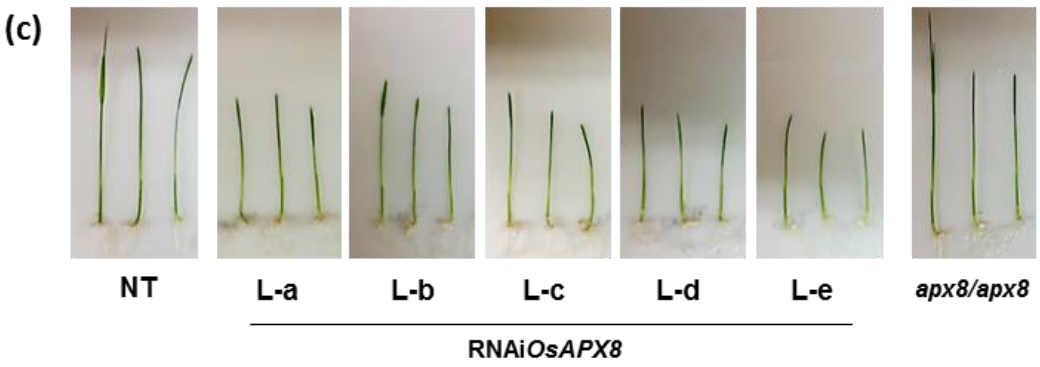


Figure 3. Chloroplastic APX activity in NT, RNAiOsAPX7 and RNAiOsAPX7/8 plants. (a) *and* ***(b)*** *Measurement of APX activity under different H_2O_2 concentration in isolated chloroplast from NT, RNAiOsAPX7 and RNAiOsAPX7/8 plants leaves. Kinetics parameters of APX activity:* ***(c)*** *V_{MAX},* ***(d)*** *K_M and* ***(e)*** *catalytic efficiency (V_{MAX}/K_M). (*) indicate populations significantly different with p<0,05.*


(a)
NT
APX8 L-a
APX8 L-b
APX8 L-c
APX8 L-d
APX8 L-e
shoot lenght (cm)
0
10
20
30
(b)
NT
apx8/apx8
shoot lenght (cm)
0
10
20
30
DAS


(c)
NT
L-a
L-b
L-c
L-d
L-e
apx8/apx8
RNAiOsAPX8

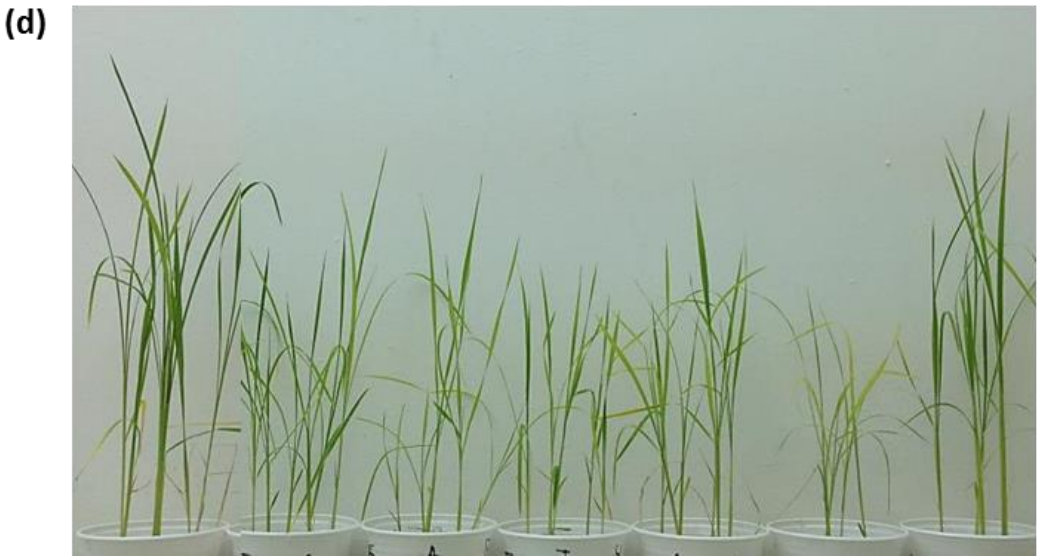

(d)

Figure 4. Growth characterization of NT (non-transformed), RNAiOsAPX8 (silenced) and apx8/apx8 (knockout) plants. (a) *Growing curve of NT and RNAiOsAPX8 plants.* ***(b)*** *Growing curve of NT and apx8/apx8 plants.* ***(c)*** *NT, RNAiOsAPX8 and apx8/apx8 seedlings with seven days old.* ***(d)*** *NT, RNAiOsAPX8 and apx8/apx8 seedlings with with four weeks old.*

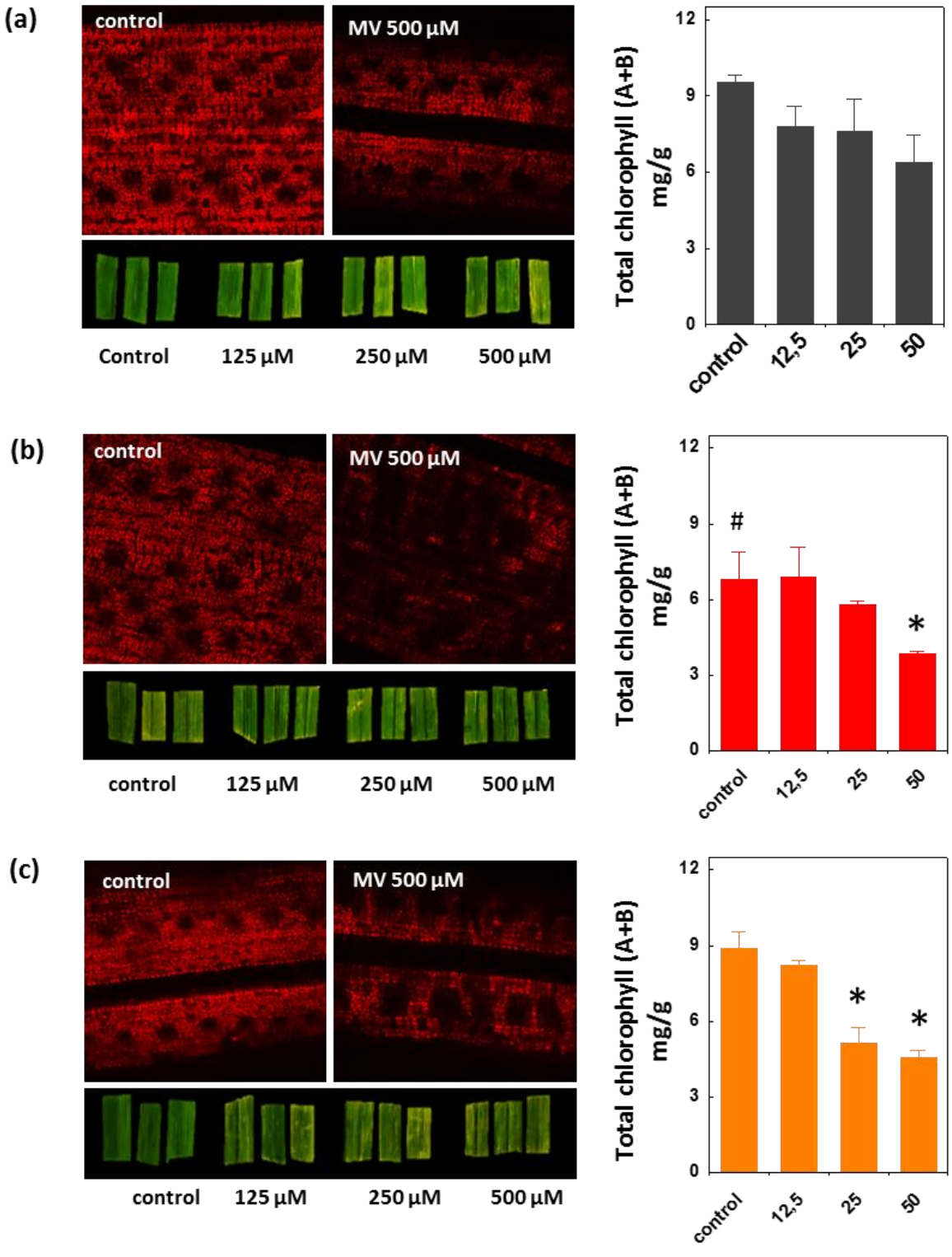

(a)
control
MV 500 µM
Control
125 µM
250 µM
500 µM
Total chlorophyll (A+B) mg/g
12
9
6
3
0
control
12,5
25
50
(b)
control
MV 500 µM
control
125 µM
250 µM
500 µM
#
*
Total chlorophyll (A+B) mg/g
12
9
6
3
0
control
12,5
25
50
(c)
control
MV 500 µM
control
125 µM
250 µM
500 µM
*
*
Total chlorophyll (A+B) mg/g
12
9
6
3
0
control
12,5
25
50

Figure 5. Effect of MV treatment in chlorophyll content of NT, RNAiOsAPX8 and apx8/apx8 plants. *The chlorophyll content was evaluated by co-focal microscopy (left) and quantified by colorimetric methods (right) in leaves from NT* ***(a)****, RNAiOsAPX8* ***(b)*** *and apx8/apx8 plants* ***(c)****. (*) indicate populations significantly different with p<0,05.*

.

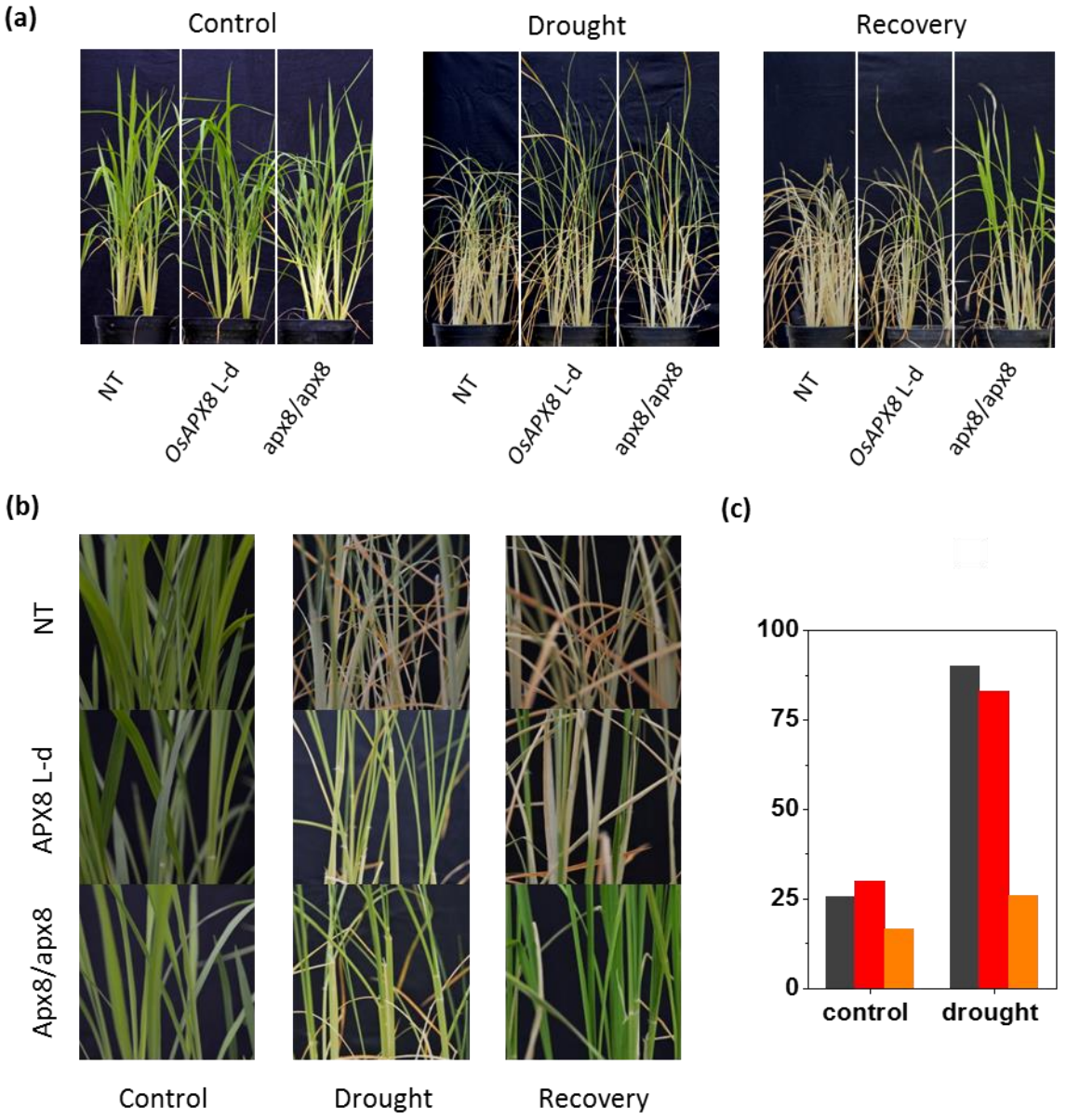


Figure 6. Effect of silencing and knockout of OsAPX8 in drought stress tolerance. (a) Drought stress treatment in NT, RNAi*OsAPX8* and *apx8/apx8* plants. One mouth-old plants (left panel) were submitted to eight day of drought (middle panel) and then then recovered by three days (right panel). **(b)** Closer pictures of plants shown in figure (a). **(c)** Membrane damage of NT (grey), RNAi*OsAPX8* (red) and apx8/apx8 plants (orange) submitted to drought stress.

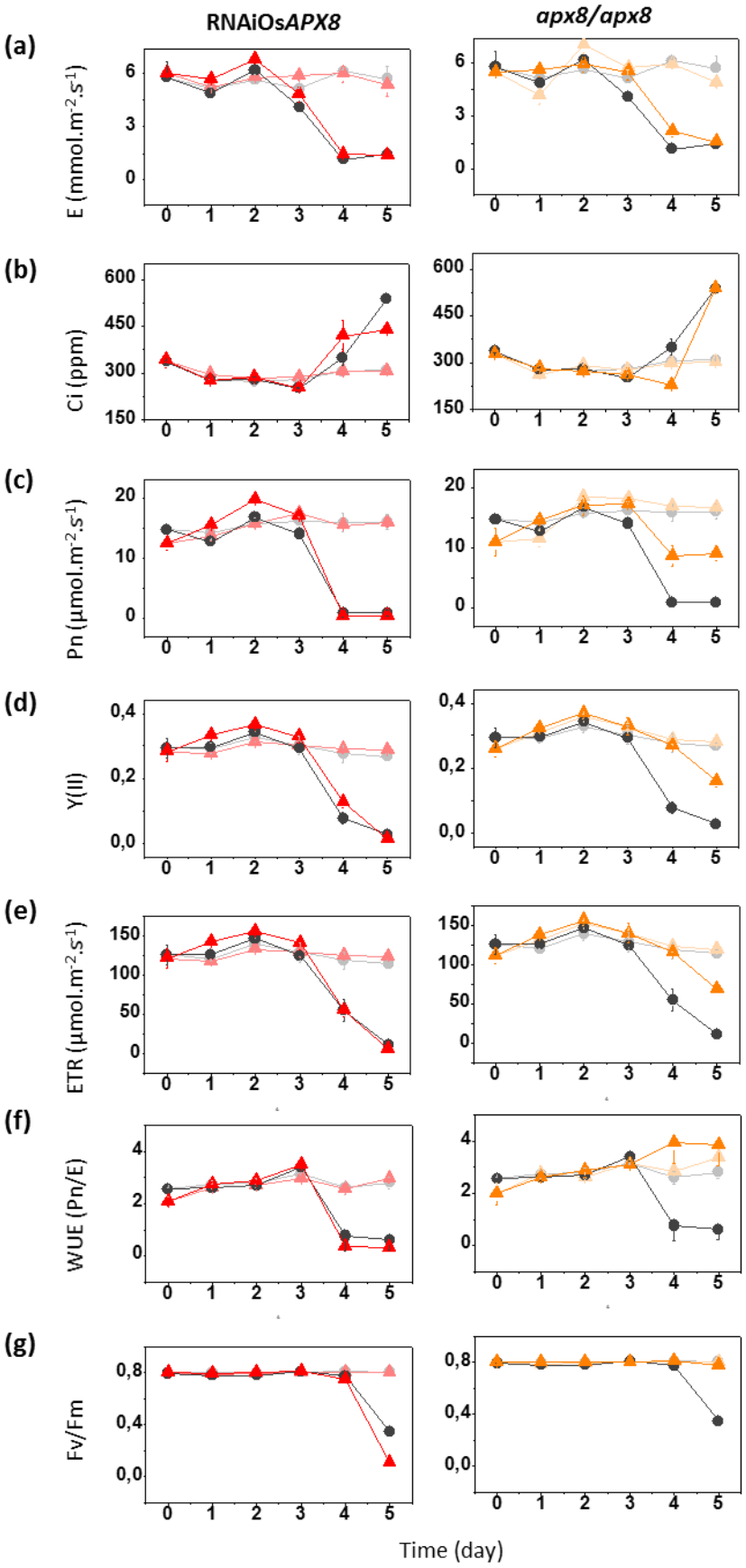


Figure 7. Photosynthetic and fluorescence parameters in plants exposed to drought stress. ***(a)*** *Transpiration,* ***(b)*** *Intracellular CO2,* ***(c)*** *Net photosynthesis,* ***(d)*** *Quantum yield of photosystem II,* ***(e)*** *Electron transport rate,* ***(f)*** *Water use efficiecy,* ***(g)*** *Potential (Fv/Fm) in leaves of NT (non-transformed) (grey), RNAiOsAPX8 (silenced) (red) and apx8/apx8 (knockout) (apx8/apx8 mutant (orange) plants. Legend: Dark grey – NT plants, Red – RNAiOsAPX8 plants, Orange – apx8/apx8 plants. The ligh colors indicate plants in normal condition. The values represent the media ± SE of at least three independent experiments*

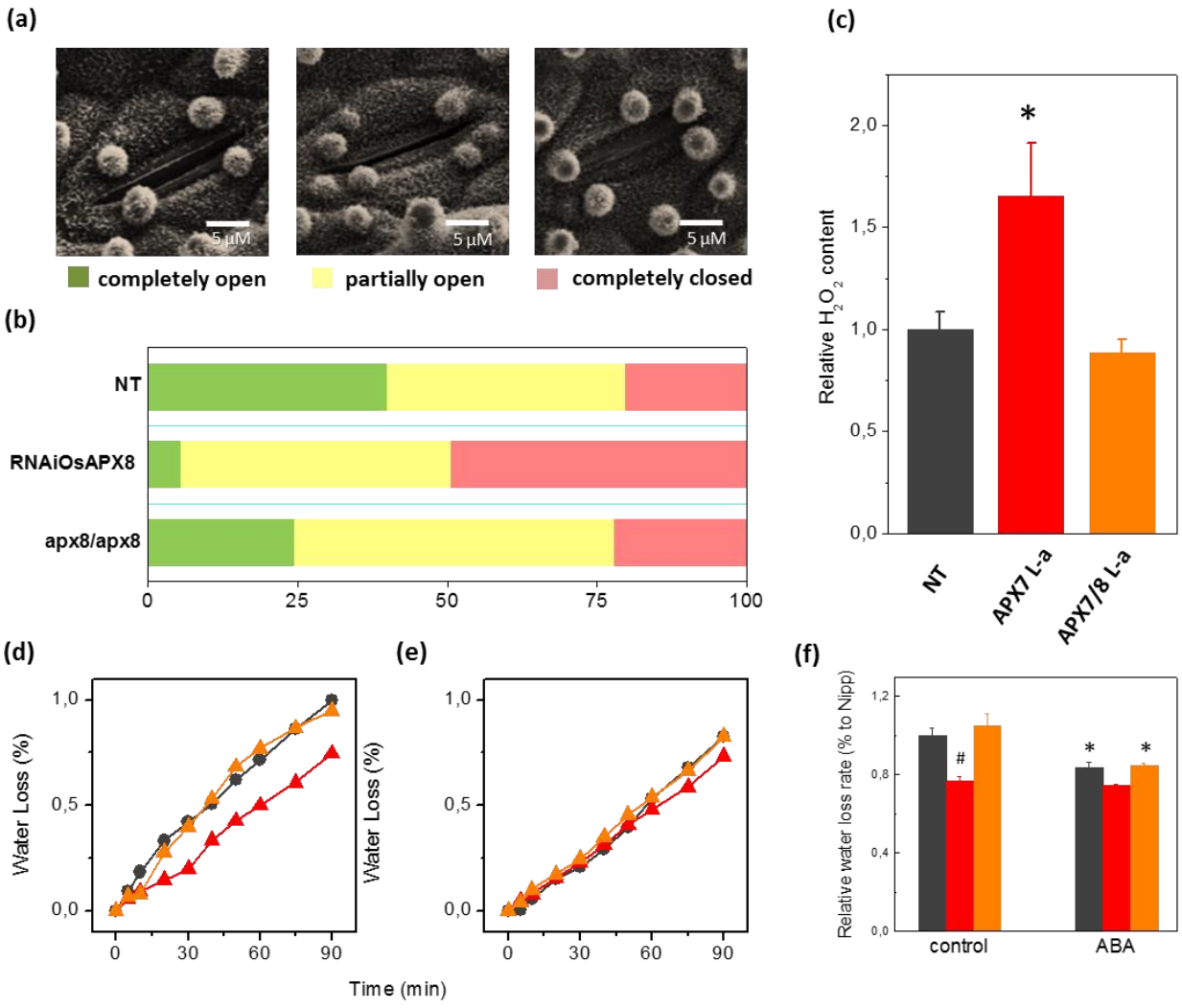

(a)
5 µM
5 µM
5 µM
completely open
partially open
completely closed
(b)
NT
RNAiOsAPX8
apx8/apx8
0
25
50
75
100
(c)
Relative H_2O_2 content
2,0
1,5
1,0
0,5
0,0
*
NT
APX7 L-a
APX7/8 L-a
(d)
Water Loss (%)
1,0
0,5
0,0
0
30
60
90
(e)
Water Loss (%)
1,0
0,5
0,0
0
30
60
90
Time (min)
(f)
Relative water loss rate (% to Nipp)
1,2
0,8
0,4
0,0
#
*
*
control
ABA

Figure 8: Effect of silencing and knockout of OsAPX8 in stomata opening and in leaf desiccation. (a) *Environmental scanning electron microscopy images of three levels of stomatal opening.* ***(b)*** *quantification of the percentage of the three levels of stomatal opening in leaves from NT, RNAiOsAPX8 and apx8/apx8 30-d-old plants (n = 200 stomata for each line).* ***(c)*** *Relative content of hydrogen peroxide in leaves from NT (grey), RNAiOsAPX8 (red) and apx8/apx8 (orange) leaves. The values represent the media ± SE of at least five independent experiments.* ***(d)*** *and* ***(e)*** *Water loss of leaves detached from plants under normal condition (treated with water) and treated with ABA 100 µM, respectively. Legend: Grey: NT, Red: RNAiOsAPX8 and Orange: apx8/apx8. For each repeat, leaves of 30-d-old plants were used in a triplicate experiment (n = 3). The water loss rate of each conditions was quantified in the figure* ***(f)****. (*) indicate populations significantly different of control situation with p<0,05, and (#) indicate populations significantly different of NT plants with p<0,05.*

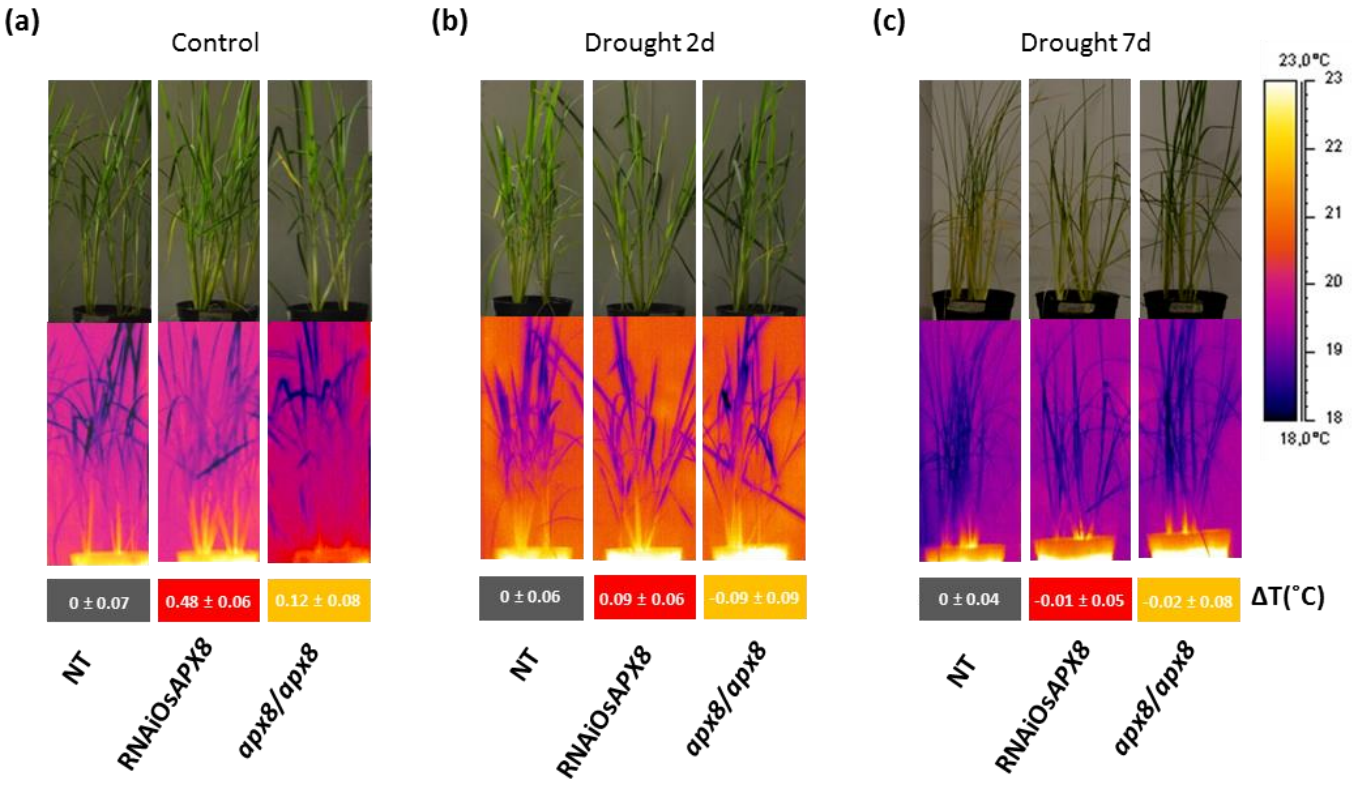
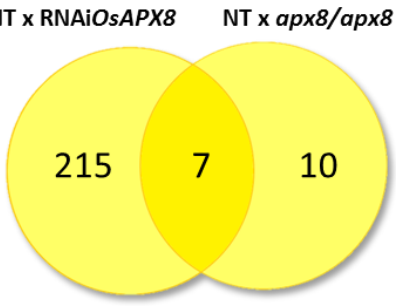


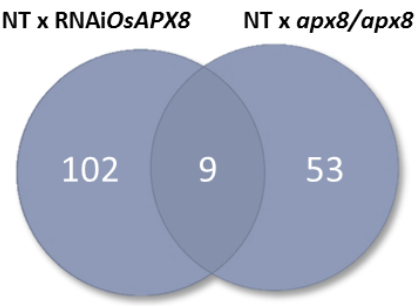
Figure 9. Thermal images of NT, RNAiOsAPX8 and apx8/apx8 plants captured by an infrared thermography device. *Thermal images of NT, RNAiOsAPX8 and apx8/apx8 plants in normal conditions* ***(a)****, under two days of drought stress* ***(b)****, and under seven days of drought stress* ***(c)****. captured by an infrared thermography device. The values represent the media ± SE of ΔT(˚C) of each line to NT plants.*

.

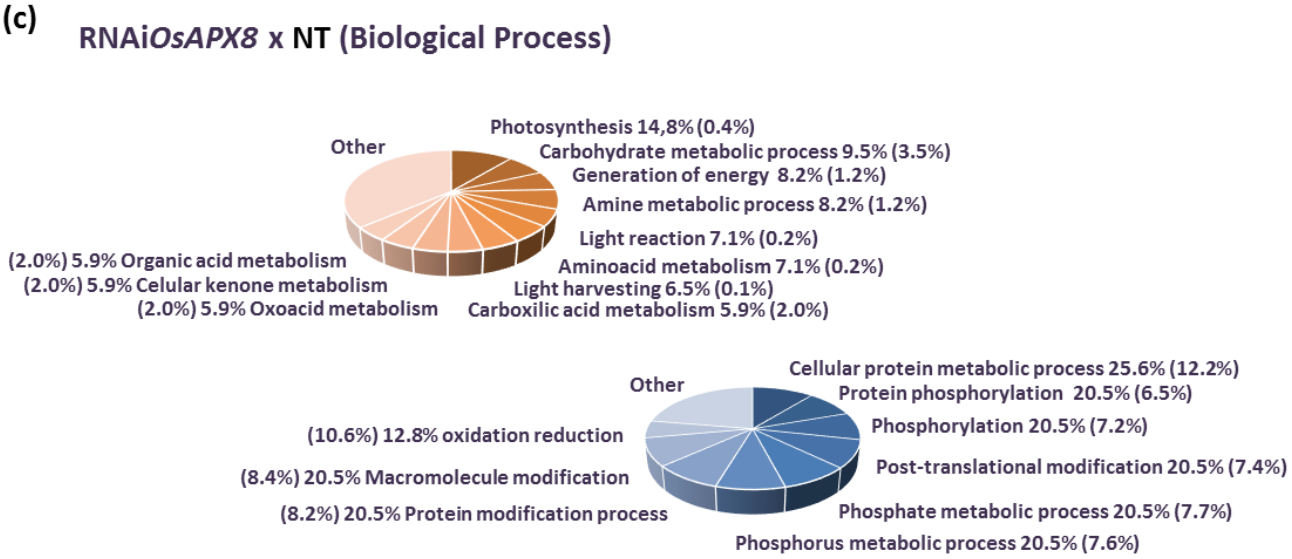
(a)


NT x RNAi*OsAPX8*
NT x *apx8/apx8*
215
7
10

(b)


NT x RNAi*OsAPX8*
NT x *apx8/apx8*
102
9
53

(c)


RNAi*OsAPX8* x NT (Biological Process)
Photosynthesis 14,8% (0.4%)
Other
Carbohydrate metabolic process 9.5% (3.5%)
Generation of energy 8.2% (1.2%)
Amine metabolic process 8.2% (1.2%)
Light reaction 7.1% (0.2%)
(2.0%) 5.9% Organic acid metabolism
Aminoacid metabolism 7.1% (0.2%)
(2.0%) 5.9% Celular kenone metabolism
Light harvesting 6.5% (0.1%)
(2.0%) 5.9% Oxoacid metabolism
Carboxilic acid metabolism 5.9% (2.0%)
Cellular protein metabolic process 25.6% (12.2%)
Other
Protein phosphorylation 20.5% (6.5%)
(10.6%) 12.8% oxidation reduction
Phosphorylation 20.5% (7.2%)
(8.4%) 20.5% Macromolecule modification
Post-translational modification 20.5% (7.4%)
(8.2%) 20.5% Protein modification process
Phosphate metabolic process 20.5% (7.7%)
Phosphorus metabolic process 20.5% (7.6%)

(d)

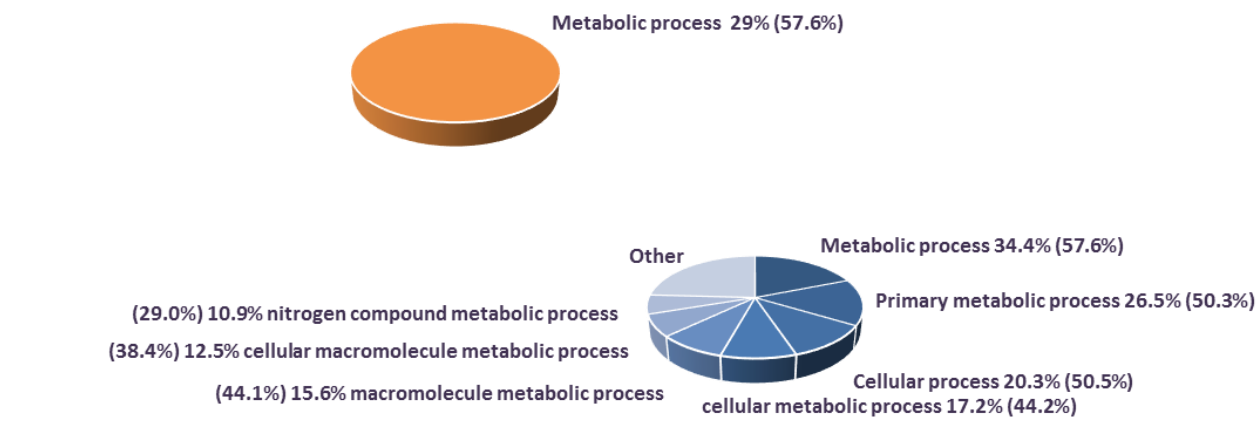
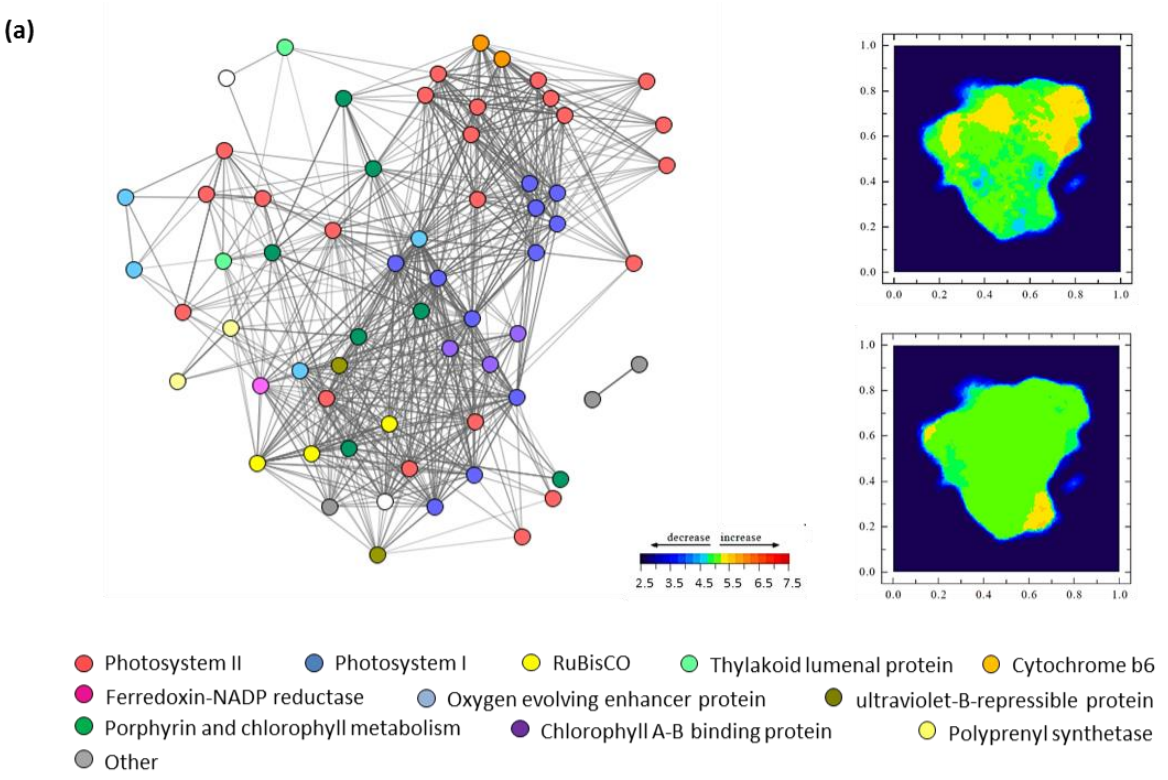

apx8/apx8 x NT (Biological Process)
Metabolic process 29% (57.6%)
Metabolic process 34.4% (57.6%)
Other
Primary metabolic process 26.5% (50.3%)
(29.0%) 10.9% nitrogen compound metabolic process
(38.4%) 12.5% cellular macromolecule metabolic process
Cellular process 20.3% (50.5%)
(44.1%) 15.6% macromolecule metabolic process
cellular metabolic process 17.2% (44.2%)

Figure 10. RNAseq analyses of gene expression in NT (non-transformed), RNAiOsAPX8 and apx8/apx8) plants. (a) *Genes up-regulated in RNAiOsAPX8 silenced and KO apx8/apx8 plants compared to NT.* ***(b)*** *Genes down-regulated in RNAiOsAPX8 silenced and KO apx8/apx8 plants compared to NT.* ***(c)*** *Ontological analysis of differentially expressed genes in RNAiOsAPX8 silenced plants compared to NT.* ***(d)*** *Ontological analysis of differentially expressed genes in apx8/apx8 KO plants compared to NT. The orange graphs indicate up-regulated genes, while the blue graphs indicate down-regulated genes. The value after the ontology indicate the percentage of genes in the total input that show the ontology and the value in brackets indicate the percentage of genes in rice genome that show the ontology.*


(a)
decrease
increase
2.5 3.5 4.5 5.5 6.5 7.5
Photosystem II
Photosystem I
RuBisCO
Thylakoid lumenal protein
Cytochrome b6
Ferredoxin-NADP reductase
Oxygen evolving enhancer protein
ultraviolet-B-repressible protein
Porphyrin and chlorophyll metabolism
Chlorophyll A-B binding protein
Polyprenyl synthetase
Other

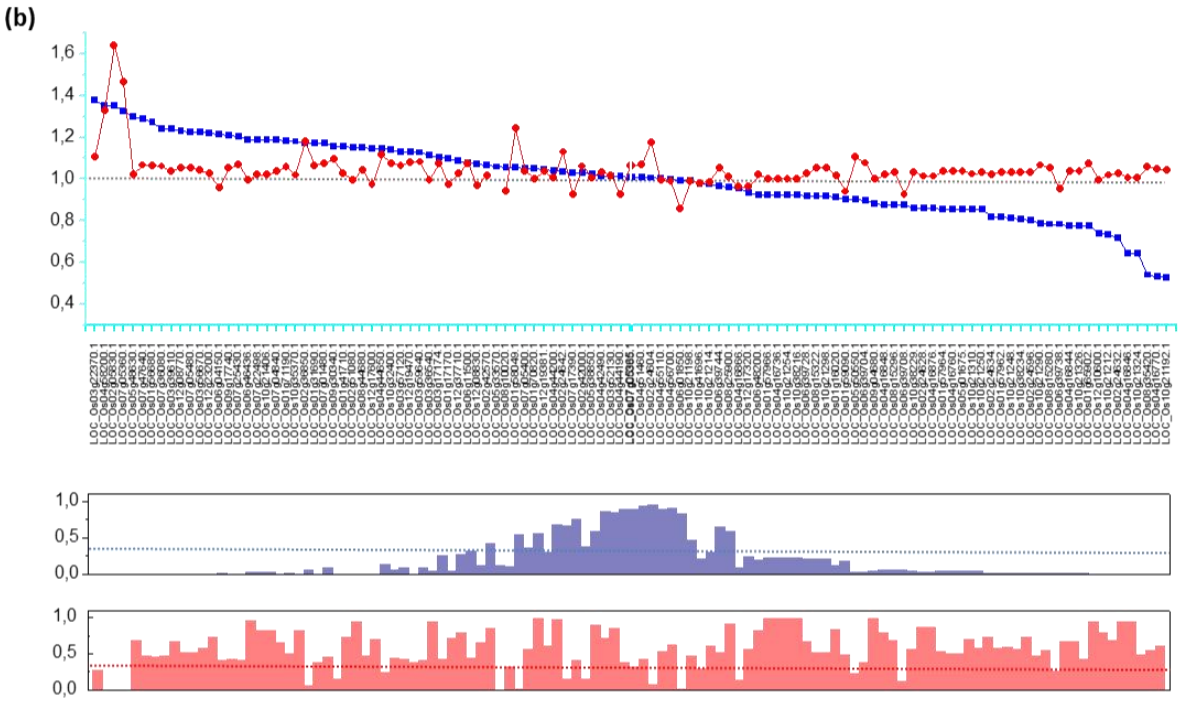

(b)
1,6
1,4
1,2
1,0
0,8
0,6
0,4
1,0
0,5
0,0
1,0
0,5
0,0

Figure 11. Effect of silencing and knockout of *OsAPX8* on rice photosynthesis gene network expression. Effect of silencing and KO of OsAPX8 on rice photosynthesis gene network expression. **(a)** Heatmap demonstrating the photosynthesis gene network expression in RNAi*OsAPX8* and *apx8/apx8* plants compared to NT (non-transformed) plants. **(b)** Relative expression of photosynthesis genes in RNAi*OsAPX8* and *apx8/apx8* plants compared to NT. Legend: Blue – RNAi*OsAPX8* x NT; Red – *apx8/apx8* x NT. The p-value of each data is indicated by blue and red bars, respectively.

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SUPPLEMENTARY FIGURE

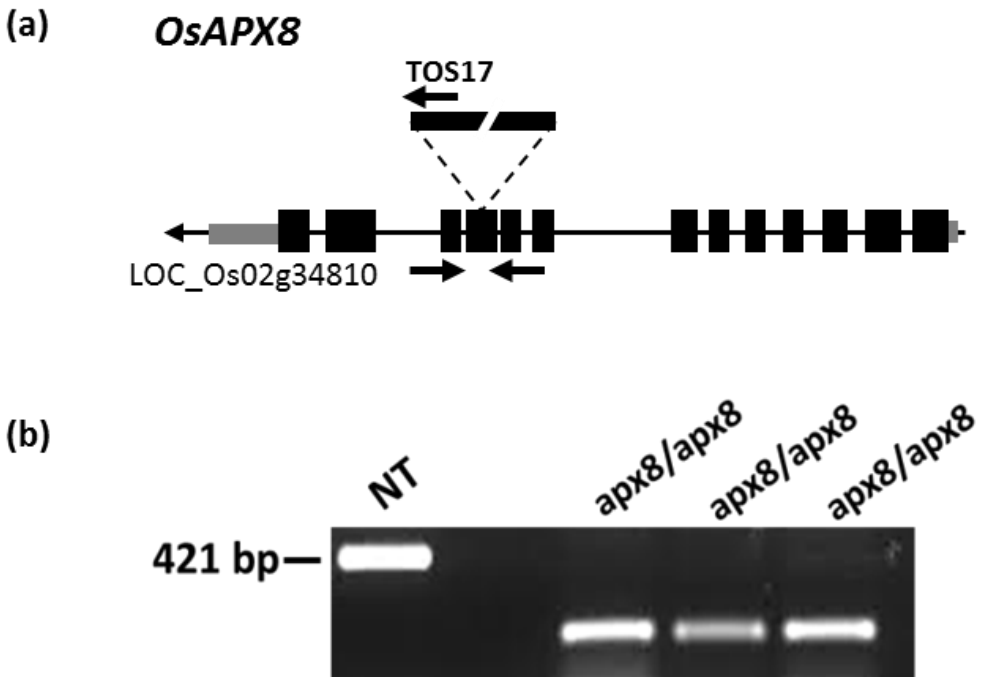

Figure S1. Genetic caracterization of apx8/apx8 (knockout) KO mutants to OsAPX8. (a) Schematic representation indicating the TOS-17 retrotransposon insertion in exon 4 of OsAPX8 gene (b) PCR demonstrating the selection of homozigous plants to TOS-17 insertion in OsAPX8 gene.

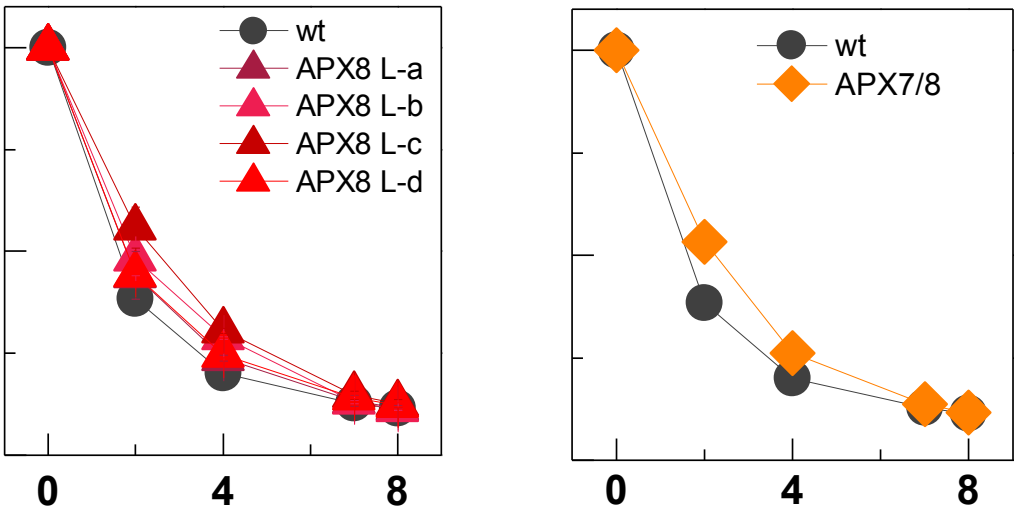

Figure S2. Relative water content in vessel of NT, RNAi*OsAPX8* and *apx8/apx8* plants submitted to drought stress. The values represent the media ± SE of at least four independent experiments

SUPPLEMENTARY TABLES

Supplementary Table S1: Sequences of primers used in RT-qPCR experiments

Oryza sativa		
APX family		
OsAPX1	F	GGTACCTCGGAGCCGCATTTCATACCAACACA
	R	CTCGAGCTACAAGGAGGCCCACCTCA
OsAPX2	F	ACCTGAGGTCCCCTTCCA
	R	CTCTCCTTGTGGCATCTTCC
OsAPX3	F	GGAGTTGTTGCCGTTGAA
	R	ACCATCAAATCCTGATCTT
OsAPX4	F	CTCGAGTGACAAGGCATTGTTGGAAG
	R	GGTACCTCGAGCAGCTGCAGCAACAGCTACC
OsAPX5	F	CTCGAGAGGGCAATCTTGGACATCTG
	R	GGTACCTCGAGGATCAAACTTTGCCCCAAGA
OsAPX6	F	CTCGAGAGGGCAATCTTGGACATCAC
	R	GGTACCTCGAGGATCAAACTTTGCTCCGAGA
OsAPX7	F	GGTACCTCGAGGACTCGTGGTCAGGAAA
	R	CTCGAGGAGCAATCTGGGTGCAAAAT
OsAPX8	F	GGTACCTCGAGAGGAGGTCATCAGACCA
	R	CTCGAGGCTGCGAAATACTCCTACCG
Other chloroplastic peroxidases		
OsAPX-R	F	GAGAAGCCACAGCCATCTT
	R	TGCATCCTTGAAGTCGGTA
OsGPX4	F	ATGGCGTCCACCACCACC
	R	GGGCTGCCTCACCATCAGTA
Reference genes		
OsFDH3	F	TTCCAATGCATTCAAAGCTG
	R	CAAAATCAGCTGGTGCTTCTC
OsUBI	F	ACCACTTGCACCGCCACTACT
	R	ACGCCTAAGCCTGCTGGTT
OsACT2	F	GGACGTACAACTGGTATCGTGTT
	R	GTTCAGCAGTGGTAGTGAAGGAG

Supplementary Table S2: Genes differently expressed (p<0.03) in RNAi *OsAPX8* and *apx8/apx8* KO mutant plants, compared to NT plants. The sublined locus indicate genes up-regulated or down-regulated in both, RNAi *OsAPX8* and *apx8/apx8* KO mutant plants

RNAi *OsAPX8* x NT plants

Up-regulated genes

	FoldChange	pvalue	Anotation
LOC_Os07g06834	7,05	9,45E-103	expressed protein
LOC_Os11g31470	3,30	1,13E-38	4350580 - expressed protein
LOC_Os10g20280	3,24	5,12E-39	transposon protein, CACTA, En/Spm sub-class
LOC_Os10g28350	2,59	5,56E-27	348646 - 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase
LOC_Os10g11250	2,42	1,12E-24	OsJ_30934 - transposon protein, CACTA, En/Spm sub-class
LOC_Os07g03040	2,38	3,59E-21	4342288 - expressed protein
LOC_Os08g04580	2,38	6,94E-24	4344638 - csAtPR5
LOC_Os03g14654	2,29	4,95E-51	4332267 - LTPL108 - Protease inhibitor/seed storage/LTP family
LOC_Os05g09724	2,06	9,39E-21	4338022 - HAD superfamily phosphatase
LOC_Os04g41620	1,99	1,40E-16	4336263 - CHIT2 - Chitinase family protein precursor
LOC_Os08g27980	1,95	3,29E-16	transposon protein, CACTA, En/Spm sub-class
LOC_Os10g39680	1,94	6,11E-13	4349267 - CHIT14 - Chitinase family protein precursor
LOC_Os01g35330	1,88	2,37E-13	circumsporozoite protein precursor
LOC_Os01g33684	1,87	1,07E-15	4323979 - disease resistance RPP13-like protein 1
LOC_Os12g36830	1,85	1,09E-11	4352487 - pathogenesis-related Bet v I family protein
LOC_Os05g12630	1,83	2,15E-15	4338118 - expressed protein
LOC_Os05g15770	1,80	1,95E-10	glycosyl hydrolase
LOC_Os12g27220	1,79	2,71E-11	OsJ_36013 - transferase family protein
LOC_Os12g27254	1,79	2,75E-11	4352177 - transferase family protein
LOC_Os04g39900	1,78	2,52E-24	4336146 - Os4bglu13 - beta-glucosidase homologue
LOC_Os11g31430	1,74	1,49E-09	4350578 - expressed protein
LOC_Os05g12640	1,73	2,18E-09	4338119 - BURP domain containing protein
LOC_Os04g41640	1,71	2,84E-09	4336264 - HEV2 - Hevein family protein precursor
LOC_Os10g31330	1,70	1,22E-12	4348767 - retrotransposon protein, unclassified
LOC_Os09g12290	1,69	4,78E-20	4346642 - bifunctional aspartokinase/homoserine dehydrogenase
LOC_Os12g43380	1,69	5,02E-10	4352852 - thaumatin
LOC_Os07g15340	1,68	1,23E-11	hAT dimerisation domain-containing protein
LOC_Os12g43430	1,68	7,73E-10	4352855 - thaumatin
LOC_Os06g11830	1,67	8,29E-12	hAT dimerisation domain-containing protein
LOC_Os08g14880	1,65	1,56E-08	transposon protein, unclassified
LOC_Os12g37260	1,62	1,57E-21	4352505 - lipoxygenase 2.1, chloroplast precursor
LOC_Os01g04050	1,62	2,84E-08	4325794 - BBTI12 - Bowman-Birk type bran trypsin inhibitor precursor,
LOC_Os05g04500	1,61	2,64E-18	4337732 - peroxidase precursor
LOC_Os02g36140	1,61	8,15E-08	4329724 - terpene synthase;
LOC_Os01g36064	1,60	1,88E-09	hAT dimerisation domain-containing protein
LOC_Os03g48030	1,60	3,77E-08	4333744 - integral membrane HPP family protein
LOC_Os03g51920	1,60	1,53E-08	4333985 - peptidase, M50 family
LOC_Os10g34930	1,60	2,04E-07	4348974 - secretory protein

LOC_Os06g45250	1,58	5,53E-07	4341760 - tRNA pseudouridine synthase family protein
LOC_Os08g17030	1,58	3,10E-10	transposon protein, CACTA, En/Spm sub-class
LOC_Os11g10710	1,57	8,81E-07	OsJ_33349 - protein kinase domain containing protein
LOC_Os09g07440	1,56	1,58E-08	retrotransposon protein, unclassified
LOC_Os01g13690	1,55	3,66E-10	4326358 - ligA
LOC_Os01g15740	1,55	2,17E-09	LOC_Os01g15740.1 - expressed protein
LOC_Os09g36700	1,54	3,34E-07	4347709 - ribonuclease T2 family domain containing protein
LOC_Os01g71670	1,54	9,07E-09	4326063 - glycosyl hydrolases family 17
LOC_Os02g52730	1,54	1,46E-06	ferredoxin--nitrite reductase
LOC_Os07g27350	1,53	2,94E-07	4343149 - atuA
LOC_Os11g31770	1,52	1,72E-11	4350599 - expressed protein
LOC_Os04g32800	1,51	2,39E-06	4335721 - basic proline-rich protein
LOC_Os04g52260	1,51	7,25E-10	4336966 - LTPL124 - Protease inhibitor/seed storage/LTP family
LOC_Os01g03340	1,50	1,28E-06	pinA - BBTI4 - Bowman-Birk type bran trypsin inhibitor precursor
LOC_Os01g03310	1,50	1,60E-09	OsJ_00194 - BBTI1 - Bowman-Birk type bran trypsin inhibitor
LOC_Os03g59190	1,50	3,96E-06	retrotransposon protein, unclassified
LOC_Os04g49194	1,50	2,33E-06	4336764 - naringenin,2-oxoglutarate 3-dioxygenase
LOC_Os09g31390	1,49	2,13E-07	4347436 - transcription factor
LOC_Os01g03320	1,49	4,40E-06	BBTI2 - Bowman-Birk type bran trypsin inhibitor precursor
LOC_Os03g01300	1,49	3,34E-08	4331302 - LTPL114 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os02g53130	1,49	2,17E-06	4330867 - nitrate reductase
LOC_Os02g50140	1,48	7,56E-06	OsJ_08287 - caleosin related protein
LOC_Os07g44670	1,48	2,22E-06	OsJ_27979 - retrotransposon protein, unclassified
LOC_Os04g05580	1,48	1,70E-05	4335018 - kinase
LOC_Os01g51570	1,48	2,03E-05	4325938 - glycosyl hydrolases family 17
LOC_Os01g06876	1,47	9,38E-06	4324407 - Cf-2
LOC_Os09g09210	1,47	1,17E-08	expressed protein
LOC_Os03g55590	1,47	1,29E-05	4334216 - MYB family transcription factor
LOC_Os06g37150	1,47	1,01E-05	4341337 - L-ascorbate oxidase precursor
LOC_Os01g28500	1,47	1,78E-05	OsJ_01863 - SCP-like extracellular protein
LOC_Os07g04240	1,45	2,43E-14	4342350 - succinate dehydrogenase flavoprotein subunit,mitochondrial precursor;
LOC_Os03g63330	1,45	3,92E-05	4334794 - aspartokinase, chloroplast precursor
LOC_Os08g35720	1,44	4,66E-05	OsJ_27571 - retrotransposon protein, unclassified
LOC_Os12g37350	1,44	7,12E-07	4352509 - lipozygenase protein
LOC_Os03g60850	1,43	5,51E-06	4334618 - peptide transporter PTR2
LOC_Os01g21250	1,43	2,98E-07	4327292 - late embryogenesis abundant protein
LOC_Os07g44250	1,43	8,26E-05	4344033 - dirigent
LOC_Os07g35510	1,43	9,08E-05	OsJ_24592 - glucan endo-1,3-beta-glucosidase precursor
LOC_Os11g42220	1,43	1,72E-08	4351006 - laccase precursor protein
LOC_Os04g09540	1,43	6,64E-05	OsJ_14006 - acyl-protein thioesterase
LOC_Os01g07170	1,43	8,12E-05	4327138 - HORMA domain containing protein
LOC_Os05g46480	1,43	5,44E-06	4326129 - late embryogenesis abundant protein, group 3
LOC_Os03g61720	1,42	1,26E-05	4334681 - glycerol-3-phosphate acyltransferase
LOC_Os07g27460	1,42	4,91E-06	4343157 - serine/threonine-protein kinase 19
LOC_Os11g17954	1,42	3,05E-06	4350282 - transposon protein, Pong sub-class
LOC_Os12g24050	1,42	5,59E-06	retrotransposon protein, unclassified
LOC_Os06g51060	1,41	0,0001253	RC24 - CHIT8 - Chitinase family protein precursor
LOC_Os01g63190	1,41	3,37E-07	4324802 - laccase precursor protein

LOC_Os07g33780	1,41	2,91E-05	4343412 - pleiotropic drug resistance protein 5
LOC_Os12g14440	1,41	7,54E-05	4351887 - Jacalin-like lectin domain containing protein
LOC_Os03g01200	1,40	9,97E-05	4331290 - SNF2 family N-terminal domain containing protein
LOC_Os06g40240	1,40	0,000118	retrotransposon protein, unclassified
LOC_Os01g36294	1,40	0,0001097	OsJ_01154 - cytochrome P450
LOC_Os04g17650	1,39	5,36E-06	OsJ_14033 - sucrose synthase
LOC_Os12g01530	1,39	2,11E-08	4351264 - ferritin-1, chloroplast precursor
LOC_Os12g12120	1,39	0,0001265	verticillium wilt disease resistance protein precursor
LOC_Os01g74450	1,39	3,37E-12	TIP1-2 - aquaporin protein
LOC_Os04g39880	1,39	6,37E-05	4336145 - Os4bglu12 - beta-glucosidase, exo-beta-glucanase
LOC_Os03g25330	1,39	0,0002221	4332925 - peroxidase precursor
LOC_Os04g32850	1,38	9,08E-08	4335725 - basic proline-rich protein
LOC_Os02g54254	1,38	7,08E-06	4330940 - saccharopine dehydrogenase
LOC_Os09g18159	1,38	0,0003264	4346821 - light repressible receptor protein kinase
LOC_Os05g38420	1,38	5,36E-06	4339004 - laccase precursor protein
LOC_Os01g15270	1,38	2,40E-05	4327719 - expressed protein
LOC_Os02g39570	1,38	7,03E-08	4329938 - ACT domain containing protein
LOC_Os03g22370	1,38	1,17E-05	4332805 - ultraviolet-B-repressible protein
LOC_Os03g58320	1,38	8,47E-05	4334428 - indole-3-glycerol phosphate lyase, chloroplast precursor
LOC_Os08g35750	1,38	0,0001147	OsJ_026468 - Cupin domain containing protein
LOC_Os05g38410	1,37	1,30E-05	4339003 - laccase precursor protein
LOC_Os12g33610	1,37	1,76E-05	phenylalanine ammonia-lyase
LOC_Os10g01044	1,37	1,17E-06	4347935 - isoflavone reductase
LOC_Os02g32520	1,37	1,63E-07	4329520 - ERD1 protein, chloroplast precursor
LOC_Os03g26530	1,37	0,0002725	4332981 - 3-ketoacyl-CoA synthase
LOC_Os04g43200	1,37	6,47E-05	4336376 - caleosin related protein
LOC_Os05g49060	1,36	6,37E-05	4339629 - uncharacterized protein ycf23
LOC_Os05g36010	1,36	0,0003185	4338899 - OsSub47 - Putative Subtilisin homologue
LOC_Os12g08090	1,36	3,96E-06	4351675 - amino acid transporter
LOC_Os02g41860	1,36	5,11E-10	4330049 - aquaporin protein
LOC_Os04g58200	1,35	2,80E-08	4337415 - protochlorophyllide reductase A, chloroplast precursor
LOC_Os06g10750	1,35	8,54E-05	4340455 - integral membrane protein DUF6 containing protein
LOC_Os03g28160	1,35	7,87E-05	OsJ_11170 - jacalin-like lectin domain containing protein
LOC_Os02g05830	1,35	0,0001344	4328326 - ribulose bisphosphate carboxylase small chain
LOC_Os11g36110	1,35	0,000235	retrotransposon protein, unclassified
LOC_Os06g01210	1,33	3,08E-11	4339833 - plastocyanin, chloroplast precursor
LOC_Os09g23300	1,33	9,69E-05	VIT1.2 - integral membrane protein
LOC_Os01g70710	1,33	9,71E-05	4326823 - heavy metal-associated domain containing protein
LOC_Os08g45030	1,33	0,000298	4346344 - multidrug resistance protein
LOC_Os01g22370	1,33	3,12E-05	4326970 - peroxidase precursor
LOC_Os07g05360	1,32	2,48E-07	4342395 - photosystem II 10 kDa polypeptide, chloroplast precursor
LOC_Os04g35520	1,32	0,0002033	4335896 - OsAPx7 - Stromal Ascorbate Peroxidase encoding gene
LOC_Os02g38240	1,31	0,0002006	4329862 - rhodanese family protein
LOC_Os05g50750	1,31	3,32E-05	4339748 - AAA family ATPase
LOC_Os12g14699	1,31	0,0002349	4351890 - protein kinase domain containing protein
LOC_Os02g02400	1,31	1,29E-06	4328073 - catalase isozyme A
LOC_Os02g55060	1,30	0,0001062	4330990 - cytochrome b5-like Heme/Steroid binding domain containing protein
LOC_Os09g36930	1,30	4,04E-07	PIP2-7 - aquaporin protein

LOC_Os07g35480	1,30	0,000299	4343487 - glucan endo-1,3-beta-glucosidase precursor
LOC_Os08g13440	1,30	6,95E-06	4344999 - cupin domain containing protein
LOC_Os05g10210	1,30	8,80E-08	4338023 - HAD superfamily phosphatase
LOC_Os09g21000	1,30	0,0001005	4346918 - potassium transporter
LOC_Os05g48630	1,30	1,66E-09	4339593 - expressed protein
LOC_Os05g34980	1,29	0,0002258	4338844 - amino acid transporter
LOC_Os01g74300	1,29	7,38E-08	MT2B - metallothionein
LOC_Os08g15840	1,29	0,0002727	4345102 - ankyrin repeat-rich protein
LOC_Os01g65690	1,29	2,62E-06	4324833 - 4,5-DOPA dioxygenase extradiol
LOC_Os11g38010	1,29	2,06E-05	4350827 - targeting protein for Xklp2
LOC_Os03g09250	1,29	2,13E-08	4331917 - inositol-3-phosphate synthase
LOC_Os08g34280	1,29	2,70E-05	4345689 - cinnamoyl-CoA reductase
LOC_Os08g02630	1,29	1,30E-05	4344539 - photosystem II core complex proteins psbY, chloroplast precursor
LOC_Os07g47640	1,28	1,75E-05	OsJ_25545 - ultraviolet-B-repressible protein
LOC_Os04g33830	1,28	4,78E-09	4335799 - membrane protein
LOC_Os03g15890	1,28	5,83E-06	4332350 - RNA recognition motif containing protein
LOC_Os02g49870	1,27	7,88E-06	4330625 - expressed protein
LOC_Os11g01530	1,27	0,0001576	4349558 - ferritin-1, chloroplast precursor
LOC_Os01g56680	1,27	1,66E-06	4327150 - photosystem II reaction center W protein, chloroplast precursor
LOC_Os08g44770	1,27	1,51E-05	4346329 - copper/zinc superoxide dismutase
LOC_Os07g11110	1,27	0,0001472	4342714 - NAD dependent epimerase/dehydratase family protein
LOC_Os11g32650	1,26	1,04E-07	4350636 - chalcone synthase
LOC_Os04g51300	1,26	1,46E-05	4336886 - peroxidase precursor
LOC_Os10g38140	1,26	3,05E-05	4349167 - glutathione S-transferase
LOC_Os02g56900	1,26	0,0002896	4331115 - thioredoxin family protein
LOC_Os07g37240	1,26	1,47E-10	4343583 - chlorophyll A-B binding protein
LOC_Os11g42500	1,26	5,33E-05	4351016 - dirigent
LOC_Os02g57120	1,25	1,47E-05	4331138 - HEAT repeat family protein
LOC_Os07g39350	1,25	8,82E-05	4343728 - transporter family protein
LOC_Os12g02320	1,25	0,0001317	4351318 - LTPL12 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os04g58710	1,25	9,88E-08	4337447 - AMP-binding domain containing protein
LOC_Os08g01380	1,25	1,09E-08	4344439 - 2Fe-2S iron-sulfur cluster binding domain containing protein
LOC_Os09g38130	1,25	0,0001015	4347808 - auxin efflux carrier component
LOC_Os06g06780	1,25	4,37E-06	4340222 - harpin-induced protein
LOC_Os07g47700	1,25	0,0001195	4344260 - NAD dependent epimerase/dehydratase family domain containing protein
LOC_Os03g34040	1,24	5,23E-05	4333233 - ribosomal protein
LOC_Os07g49110	1,24	5,14E-05	4344370 - D-alanine--D-alanine ligase family
LOC_Os07g37550	1,24	5,47E-09	4343604 - chlorophyll A-B binding protein
LOC_Os01g52240	1,24	3,59E-05	OsJ_02493 - chlorophyll A-B binding protein
LOC_Os07g36080	1,24	1,63E-08	4343515 - oxygen evolving enhancer protein 3 domain containing protein
LOC_Os03g39610	1,24	1,10E-08	4333359 - chlorophyll A-B binding protein
LOC_Os06g46930	1,24	0,0001849	4341869 - ribosomal protein L24
LOC_Os03g61260	1,24	0,0001271	4334651 - ribosomal L18p/L5e family protein; Binds 5S rRNA
LOC_Os03g27120	1,24	0,0001107	4333009 - ICE-like protease p20 domain containing protein
LOC_Os06g21590	1,23	5,38E-09	4340892 - chlorophyll A-B binding protein
LOC_Os03g56950	1,23	0,0001997	4334324 - phytochrome-interacting factor 4
LOC_Os09g25850	1,23	0,0001209	4347115 - WAX2

LOC_Os12g37840	1,23	0,0002666	4352546 - boron transporter protein
LOC_Os03g15020	1,23	0,0002352	4332289 - beta-galactosidase precursor
LOC_Os04g53190	1,23	4,25E-06	4337046 - CPuORF12 - conserved peptide uORF-containing transcript
LOC_Os12g08770	1,23	9,72E-07	4351694 - photosystem I reaction center subunit N, chloroplast precursor
LOC_Os01g55570	1,23	7,02E-05	4327018 - expressed protein
LOC_Os07g05480	1,23	7,55E-07	4342404 - photosystem I reaction center subunit, chloroplast precursor
LOC_Os04g53195	1,23	9,46E-06	expressed protein
LOC_Os12g42980	1,22	0,0003219	4352839 - cysteine synthase
LOC_Os03g56670	1,22	1,57E-06	4334300 - photosystem I reaction center subunit III, chloroplast precursor
LOC_Os02g37690	1,22	0,000239	4329821 - UDP-glucoronosyl and UDP-glucosyl transferase
LOC_Os01g02870	1,22	0,0002816	4326073 - auxin-induced protein 5NG4
LOC_Os12g23200	1,22	2,27E-05	4352085 - photosystem I reaction center subunit XI, chloroplast precursor
LOC_Os04g38410	1,22	9,38E-07	4336028 - chlorophyll A-B binding protein
LOC_Os06g15400	1,21	2,57E-06	4340704 - expressed protein
LOC_Os06g18670	1,21	0,000224	4340788 - anthocyanidin 3-O-glucosyltransferase
LOC_Os09g17740	1,21	1,42E-05	4346803 - chlorophyll A-B binding protein
LOC_Os03g63480	1,20	0,0002579	4334805 - ankyrin repeat domain containing protein
LOC_Os07g25430	1,20	7,28E-06	photosystem I reaction center subunit IV A, chloroplast precursor
LOC_Os05g03040	1,20	0,0001696	AP2D23 - AP2 domain containing protein
LOC_Os11g13890	1,20	1,93E-07	4350176 - chlorophyll A-B binding protein
LOC_Os01g05650	1,20	0,000283	4324514 - metallothionein;
LOC_Os08g33820	1,20	2,68E-07	4345663 - chlorophyll A-B binding protein
LOC_Os06g24070	1,20	0,0002163	4340977 - myb-like DNA-binding domain containing protein
LOC_Os10g28360	1,20	5,51E-05	4348647 - 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase protein
LOC_Os04g28620	1,19	8,12E-05	4335562 - male sterility protein
LOC_Os02g15750	1,19	0,0001341	4328925 - expressed protein
LOC_Os02g36570	1,19	5,26E-05	4329751 - ABC1 family domain containing protein
LOC_Os03g53800	1,19	1,10E-05	4334114 - periplasmic beta-glucosidase precursor
LOC_Os07g04840	1,18	4,73E-06	4342370 - PsbP
LOC_Os03g36750	1,18	0,000308	4333266 - cbbY
LOC_Os07g38960	1,18	2,52E-06	4343709 - chlorophyll A-B binding protein
LOC_Os01g18800	1,18	7,95E-05	4325161 - CAMK_KIN1/SNF1/Nim1_like.9
LOC_Os06g36090	1,18	8,50E-05	4341281 - ABC-2 type transporter
LOC_Os08g43400	1,17	0,0001101	4346235 - kinesin motor domain containing protein
LOC_Os01g31690	1,17	2,89E-05	oxygen-evolving enhancer protein 1, chloroplast precursor
LOC_Os03g01014	1,17	0,0001952	expressed protein
LOC_Os07g49400	1,16	7,38E-05	4344397 - OsAPx2 - Cytosolic Ascorbate Peroxidase
LOC_Os09g30340	1,16	0,0001957	4347395 - photosystem I reaction center subunit
LOC_Os02g10390	1,15	3,86E-05	4328623 - chlorophyll A-B binding protein
LOC_Os08g06100	1,15	0,0001359	4344702 - O-methyltransferase
LOC_Os12g08760	1,15	8,35E-05	4351693 - carboxyvinyl-carboxyphosphonate phosphorylmutase
LOC_Os08g44680	1,15	0,0001339	4346326 - photosystem I reaction center subunit II

Down-regulated genes

	FoldChange	pvalue	Anotation
LOC_Os01g72370	0,43	1,53E-20	4325750 - helix-loop-helix DNA-binding domain containing protein
LOC_Os10g21192	0,53	1,71E-25	photosystem Q
LOC_Os10g39880	0,53	4,80E-15	photosynthetic reaction center protein

LOC_Os04g16770	0,53	4,93E-28	photosynthetic reaction center protein
LOC_Os02g36264	0,54	5,99E-12	terpene synthase;
LOC_Os04g27670	0,54	1,55E-13	4335526 - terpene synthase family
LOC_Os08g35420	0,54	7,62E-29	photosynthetic reaction center protein
LOC_Os04g27190	0,57	5,02E-18	4335518 - terpene synthase
LOC_Os04g27340	0,57	3,34E-20	OsJ_14329 - terpene synthase
LOC_Os12g12470	0,58	7,55E-15	4351814 - NADP-dependent oxidoreductase
LOC_Os08g44360	0,59	1,32E-10	4346301 - male sterility protein 2
LOC_Os11g29990	0,59	6,65E-10	NBS-LRR type disease resistance protein
LOC_Os10g05820	0,61	1,24E-09	4348114 - POEI5 - Pollen Ole e I allergen and extensin family protein
LOC_Os09g19229	0,61	6,10E-09	4346837 - protein kinase domain containing protein
LOC_Os11g17330	0,62	1,02E-07	OsJ_33608 - stripe rust resistance protein Yr10
LOC_Os10g05930	0,62	6,03E-08	4348118 - POEI10 - Pollen Ole e I allergen and extensin family
LOC_Os08g44040	0,62	2,05E-12	4346275 - rhamnogalacturonate lyase
LOC_Os01g45914	0,62	5,78E-10	expressed protein
LOC_Os09g08990	0,62	3,30E-09	4346556 - cytochrome P450
LOC_Os11g01010	0,63	1,80E-10	ATG8D - autophagy-related protein 8D
LOC_Os01g57968	0,63	1,26E-07	expressed protein
LOC_Os12g10580	0,64	7,15E-07	rps15 - ribulose bisphosphate carboxylase large chain
LOC_Os01g18120	0,64	4,92E-07	OsJ_01338 - cinnamoyl CoA reductase
LOC_Os04g29680	0,64	3,06E-07	4335581 - OsWAK38 - OsWAK receptor-like protein kinase
LOC_Os01g57942	0,64	1,60E-06	cytochrome b6
LOC_Os04g16846	0,64	1,60E-06	cytochrome b6
LOC_Os10g21324	0,64	1,60E-06	cytochrome b6
LOC_Os07g37385	0,65	4,60E-07	OsJ_24732 - LTPL109 - Protease inhibitor/seed storage/LTP family
LOC_Os07g03880	0,65	1,49E-07	4342330 - lectin-like receptor kinase
LOC_Os11g36960	0,65	1,51E-06	4350772 - dnaJ domain containing protein
LOC_Os10g04730	0,66	2,61E-09	4348065 - TKL_IRAK_DUF26-la.6 - DUF26 kinases
LOC_Os09g17560	0,66	4,98E-06	4346795 - O-methyltransferase
LOC_Os04g29770	0,66	5,17E-09	OsJ_14438 - wall-associated receptor kinase-like 3
LOC_Os06g38480	0,68	1,897E-05	4341388 - retrotransposon protein
LOC_Os04g10940	0,68	1,054E-05	expressed protein
LOC_Os05g25370	0,68	1,642E-05	TKL_IRAK_CrRLK1L-1.2
LOC_Os06g49840	0,68	1,012E-05	4342047 - OsMADS16 - MADS-box family gene with MIKCc type-box
LOC_Os04g28820	0,68	6,381E-06	4335569 - retrotransposon protein, unclassified
LOC_Os10g21190	0,69	4,247E-05	OsJ_31208 - expressed protein
LOC_Os10g05860	0,69	1,901E-06	OsJ_30736 - POEI7 - Pollen Ole e I allergen and extensin family
LOC_Os04g16734	0,69	5,077E-05	maturase K
LOC_Os01g60880	0,69	6,683E-05	transposon protein, unclassified
LOC_Os03g14130	0,70	1,719E-06	4332232 - POEI1 - Pollen Ole e I allergen and extensin family protein
LOC_Os04g30030	0,70	0,0001006	4335588 - cysteine-rich receptor-like protein kinase 12 precursor
LOC_Os04g16874	0,71	0,000146	photosystem II 44 kDa reaction center protein
LOC_Os10g21194	0,71	8,147E-05	maturase K
LOC_Os11g38580	0,71	0,0001787	4350851 - NBS-LRR type disease resistance protein
LOC_Os11g13750	0,71	1,563E-05	4350169 - expressed protein
LOC_Os10g05880	0,71	1,406E-05	4348115 - POEI8 - Pollen Ole e I allergen and extensin family
LOC_Os07g41250	0,71	1,202E-05	4343841 - peptide transporter PTR2
LOC_Os02g11730	0,72	0,0002187	transposon protein

LOC_Os02g18360	0,72	0,0002629	transposon protein
LOC_Os04g34290	0,72	0,0002292	protein kinase
LOC_Os11g02330	0,72	0,0002067	4349600 - LTPL22 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os02g24632	0,72	0,0002941	photosystem II 44 kDa reaction center protein
LOC_Os09g27420	0,72	5,689E-08	4347204 - formate--tetrahydrofolate ligase
LOC_Os04g10924	0,72	0,0001079	OsJ_13943 - hypothetical protein
LOC_Os08g15236	0,72	0,0002445	uncharacterized protein ycf68
LOC_Os02g36210	0,73	0,0003277	4329727 - ent-kaurene synthase
LOC_Os12g33050	0,73	0,0001301	transposon protein, CACTA, En/Spm sub-class
LOC_Os11g20330	0,73	0,0001145	OsJ_33738 - expressed protein
LOC_Os10g09990	0,73	0,0003195	4348200 - cytokinin-O-glucosyltransferase 3
LOC_Os07g03000	0,73	4,771E-05	OsJ_22922 - receptor-like protein kinase precursor
LOC_Os01g03490	0,73	2,059E-05	4326005 - heavy-metal-associated domain-containing protein
LOC_Os01g20200	0,73	7,091E-05	transposon protein, CACTA, En/Spm sub-class
LOC_Os12g02290	0,73	0,0002759	4351314 - LTPL23 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os07g46080	0,73	0,0001935	OsJ_33983 - conserved hypothetical protein
LOC_Os11g27329	0,74	0,0002044	4350473 - OsSCP62 - Putative Serine Carboxypeptidase homologue
LOC_Os12g10600	0,74	0,0001929	photosystem II P680 chlorophyll A apoprotein
LOC_Os07g26660	0,74	0,0001341	PIP2-5 - aquaporin protein
LOC_Os02g46680	0,74	9,816E-05	4330390 - multidrug resistance protein
LOC_Os01g05900	0,74	0,0001182	4324495 - Core histone H2A/H2B/H3/H4 domain containing protein
LOC_Os01g55410	0,74	0,0001235	OsJ_03519 - expressed protein
LOC_Os08g14940	0,75	1,249E-06	4345064 - receptor kinase
LOC_Os09g15480	0,75	1,241E-05	4346725 - Ser/Thr-rich protein T10 in DGCR region
LOC_Os10g05840	0,75	7,385E-05	POEI6 - Pollen Ole e I allergen and extensin family protein
LOC_Os09g25340	0,75	0,0002476	transposon protein, CACTA, En/Spm sub-class
LOC_Os12g13340	0,75	3,178E-05	OsJ_35667 - expressed protein
LOC_Os02g20360	0,76	8,383E-08	OsNAAT1 - tyrosine aminotransferase
LOC_Os02g30360	0,76	0,0001458	LOC_Os02g30360.1 - transposon protein, CACTA, En/Spm sub-class
LOC_Os01g38359	0,76	7,516E-06	4327925 - peptidyl-prolyl cis-trans isomerase, FKBP-type
LOC_Os07g08290	0,76	2,015E-06	4342561 - expressed protein
LOC_Os01g15830	0,77	1,861E-07	4325129 - peroxidase precursor
LOC_Os05g11320	0,77	1,861E-11	4338063 - metallothionein-like protein 3B
LOC_Os01g47400	0,77	7,581E-05	4327860 - OsMan01 - Endo-Beta-Mannanase
LOC_Os04g11400	0,77	2,075E-05	4335124 - expressed protein
LOC_Os04g31070	0,77	3,76E-06	4335627 - acyl-desaturase
LOC_Os11g10630	0,78	0,0001299	retrotransposon protein
LOC_Os01g57340	0,78	0,0001858	4327844 - rp1
LOC_Os09g02840	0,78	0,0001154	transposon protein, CACTA, En/Spm sub-class
LOC_Os01g07590	0,78	0,0001174	universal stress protein domain containing protein
LOC_Os02g13510	0,79	3,086E-07	receptor-like protein kinase 5 precursor
LOC_Os02g58150	0,79	0,0003298	expressed protein
LOC_Os04g39150	0,79	3,28E-06	pathogenesis-related Bet v I family protein
LOC_Os04g21820	0,79	0,0003138	OsJ_14137 - OsWAK33 - OsWAK receptor-like protein OsWAK-RLP
LOC_Os07g31720	0,80	9,762E-10	GTPase activating protein
LOC_Os03g29760	0,80	0,0002826	nuclear transcription factor Y subunit
LOC_Os11g10310	0,80	9,389E-05	receptor-like protein kinase 2 precursor
LOC_Os07g48780	0,81	5,804E-05	4324384 - OsCam1-2 - Calmodulin

LOC_Os02g43500	0,81	1,027E-06	4330168 - OR
LOC_Os09g36220	0,81	6,869E-07	4347674 - response regulator receiver domain containing protein
LOC_Os02g40260	0,81	1,407E-08	OsJ_07530 - uncharacterized protein At4g06744 precursor
LOC_Os03g10090	0,83	6,168E-05	4331945 - transporter family protein
LOC_Os04g47360	0,83	6,77E-05	4336657 - OsPOP9 - Putative Prolyl Oligopeptidase homologue
LOC_Os05g07860	0,84	7,964E-05	4337940 - uvrB/uvrC motif family protein
LOC_Os09g18594	0,84	1,856E-05	4346832 - protein kinase domain containing protein
LOC_Os04g37690	0,84	9,537E-05	4335992 - RNA recognition motif containing protein
LOC_Os01g37910	0,84	1,235E-06	OsJ_02232 - vacuolar-processing enzyme precursor
LOC_Os03g06240	0,85	6,091E-05	4331683 - YT521-B-like family domain containing protein
LOC_Os12g12850	0,86	1,759E-05	4351828 - ATP-dependent Clp protease ATP-binding subunit clpA homolog
LOC_Os07g44330	0,87	0,0001695	4344039 - kinase

apx8/apx8 x NT plants

Up-regulated genes

	FoldChange	pvalue	Anotation
LOC_Os03g30740	1,92	1,00E-12	4333130 - expressed protein
LOC_Os08g14880	1,72	5,54E-09	transposon protein, unclassified
LOC_Os02g05830	1,64	1,44E-09	4328326 - ribulose bisphosphate carboxylase small chain
LOC_Os04g02490	1,64	1,83E-10	4334942 - hypothetical protein
LOC_Os08g10310	1,63	4,32E-11	4344914 - SHR5-receptor-like kinase
LOC_Os07g26100	1,63	2,38E-10	4343097 - expressed protein
LOC_Os08g27170	1,59	1,33E-06	OsJ_27047 - calmodulin binding protein
LOC_Os02g35970	1,58	1,27E-06	4329720 - BTBN2 - Bric-a-Brac, Tramtrack
LOC_Os03g14654	1,55	5,86E-07	4332267 - LTPL108 - Protease inhibitor/seed storage/LTP family protein
LOC_Os02g06300	1,54	2,45E-06	B1103G11.7 - GTP-binding protein lepA
LOC_Os05g12640	1,54	1,54E-06	4338119 - BURP domain containing protein
LOC_Os01g07170	1,51	1,19E-05	4327138 - HORMA domain containing protein
LOC_Os07g05360	1,46	2,254E-06	4342395 - photosystem II 10 kDa polypeptide
LOC_Os04g18770	1,46	1,639E-07	retrotransposon protein
LOC_Os11g31470	1,45	2,502E-05	4350580 - expressed protein
LOC_Os11g10520	1,43	3,791E-05	4350055 - dehydrogenase
LOC_Os12g24790	1,37	2,355E-05	retrotransposon protein, LINE subclass

Down-regulated genes

	FoldChange	pvalue	Anotation
LOC_Os02g34810	0,23	2,57E-66	4329643 - OsAPx8 - Thylakoid-bound Ascorbate Peroxidase
LOC_Os10g29650	0,40	1,03E-22	4344069 - retrotransposon protein
LOC_Os11g17330	0,52	4,30E-12	OsJ_33608 - stripe rust resistance protein Yr10
LOC_Os10g05820	0,54	3,71E-11	4348114 - POEI5 - Pollen Ole e I allergen and extensin family protein
LOC_Os10g05860	0,56	5,95E-10	OsJ_30736 - POEI7 - Pollen Ole e I allergen and extensin family protein
LOC_Os02g02930	0,58	9,633E-09	4328124 - terpene synthase
LOC_Os10g05840	0,58	1,441E-08	POEI6 - Pollen Ole e I allergen and extensin family protein
LOC_Os09g20390	0,59	3,848E-08	OsJ_29113 - uncharacterized glycosyl hydrolase Rv2006/MT2062
LOC_Os07g31690	0,60	2,947E-08	retrotransposon protein
LOC_Os11g29990	0,61	1,294E-08	NBS-LRR type disease resistance protein
LOC_Os10g31320	0,62	4,757E-07	4348766 - retrotransposon protein, unclassified

LOC_Os02g34760	0,62	4,95E-08	OsJ_07110 - retrotransposon protein, unclassified
LOC_Os03g44880	0,63	8,227E-07	4333595 - Cupin domain containing protein
LOC_Os10g05970	0,63	8,067E-15	4348120 - POEI12 - Pollen Ole e I allergen and extensin family protein precursor
LOC_Os02g20540	0,63	6,743E-09	4329121 - fasciclin domain containing protein
LOC_Os02g30910	0,64	6,788E-07	4329464 - nodulin MtN3 family protein
LOC_Os07g10850	0,64	7,784E-08	retrotransposon protein, unclassified
LOC_Os09g33470	0,65	4,175E-08	mitochondrial carrier protein
LOC_Os02g15690	0,65	5,764E-07	4328920 - polygalacturonase
LOC_Os02g40260	0,65	1,328E-12	OsJ_07530 - uncharacterized protein At4g06744 precursor
LOC_Os08g35760	0,66	8,838E-09	4345763 - Cupin domain containing protein
LOC_Os02g32814	0,66	2,97E-10	4329543 - heavy metal-associated domain containing protein
LOC_Os10g05990	0,66	7,674E-14	4348122 - POEI14 - Pollen Ole e I allergen and extensin family protein
LOC_Os10g05980	0,66	3,284E-13	4348121 - POEI13 - Pollen Ole e I allergen and extensin family protein
LOC_Os03g58320	0,66	9,065E-06	4334428 - indole-3-glycerol phosphate lyase, chloroplast precursor
LOC_Os12g02300	0,67	4,016E-11	4349601 - LTPL26 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os06g35970	0,67	1,868E-08	4341274 - meiosis 5
LOC_Os01g05730	0,67	1,122E-06	4329640 - retrotransposon protein, unclassified
LOC_Os01g45060	0,68	1,908E-05	polygalacturonase
LOC_Os11g02350	0,68	1,007E-07	LTPL25 - Protease inhibitor/seed storage/LTP family protein precursor
LOC_Os12g05080	0,68	4,358E-05	4351490 - PMR5
LOC_Os12g05290	0,68	9,837E-07	retrotransposon protein, unclassified
LOC_Os10g05750	0,68	5,028E-07	4348110 - POEI3 - Pollen Ole e I allergen and extensin family protein precursor
LOC_Os07g03880	0,68	2,103E-05	4342330 - lectin-like receptor kinase
LOC_Os09g08990	0,68	7,926E-06	4346556 - cytochrome P450
LOC_Os09g07290	0,68	3,081E-05	4346496 - GDSL-like lipase/acylhydrolase
LOC_Os06g37300	0,68	2,084E-05	4341343 - cytochrome P450
LOC_Os07g37850	0,69	1,027E-07	4343628 - expressed protein
LOC_Os07g15340	0,69	1,157E-05	hAT dimerisation domain-containing protein
LOC_Os09g34250	0,69	2,097E-05	4347593 - UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein
LOC_Os10g06000	0,69	1,167E-08	4348123 - POEI15 - Pollen Ole e I allergen and extensin family protein precursor
LOC_Os01g44970	0,69	7,566E-08	4326573 - polygalacturonase
LOC_Os12g36880	0,69	1,295E-07	4352491 - pathogenesis-related Bet v I family protein
LOC_Os07g46480	0,69	6,311E-06	4344165 - eukaryotic aspartyl protease domain containing protein
LOC_Os06g11830	0,69	1,089E-05	hAT dimerisation domain-containing protein
LOC_Os01g36064	0,70	1,81E-05	hAT dimerisation domain-containing protein
LOC_Os01g18860	0,71	2,825E-06	S-adenosylmethionine synthetase
LOC_Os10g02880	0,71	2,374E-05	4347994 - O-methyltransferase
LOC_Os09g15480	0,72	3,581E-07	4346725 - Ser/Thr-rich protein T10 in DGCR region
LOC_Os07g34260	0,72	1,881E-05	4343432 - chalcone and stilbene synthases
LOC_Os02g48770	0,72	7,776E-06	4330542 - SAM dependent carboxyl methyltransferase
LOC_Os01g52770	0,72	1,304E-05	OsJ_03329 - GDSL-like lipase/acylhydrolase
LOC_Os06g49350	0,72	2,114E-05	OsJ_22605 - retrotransposon protein, unclassified
LOC_Os03g01800	0,72	1,705E-06	4331336 - glycosyl hydrolases family 16
LOC_Os04g39150	0,73	2,844E-06	4336091 - pathogenesis-related Bet v I family protein
LOC_Os01g55000	0,73	2,421E-06	expressed protein
LOC_Os03g04680	0,73	1,351E-05	4331571 - cytochrome P450
LOC_Os01g72290	0,73	1,275E-05	4325743 - expressed protein

LOC_Os01g71840	0,73	2,915E-05	P0466H10.8 - retrotransposon protein, unclassified
LOC_Os05g04820	0,73	1,221E-06	4337756 - MYB family transcription factor
LOC_Os08g44270	0,73	5,678E-06	4346292 - vignain precursor
LOC_Os01g58550	0,74	2,728E-05	4327631 - methyladenine glycosylase
LOC_Os01g43710	0,77	1,203E-05	4326657 - cytochrome P450 72A1
LOC_Os03g22634	0,77	2,43E-05	OsJ_10830 - terpene synthase
LOC_Os03g51650	0,79	6,951E-06	4333970 - membrane protein

3.5 CAPÍTULO 5

IDENTIFICAÇÃO DE FATORES DE TRANSCRIÇÂO QUE SE LIGUEM À REGIAO PROMOTORA DE *OsAPX8* E POTENCILAMENTE PODEM REGULAR A EXPRESSÃO GÊNICA

Conforme demonstrado nos capítulos anteriores, o gene *OsAPX8* desempenha um importante papel no controle da abertura estomática e na tolerância de plantas ao estresse de seca. Desta forma, a identificação de fatores de transcrição que regulam a expressão de *OsAPX8* pode fornecer dados importantes a respeito de mecanismos regulatórios mediadores de tais fenômenos. Neste capítulo, que corresponde ao trabalho desenvolvido no período de doutorado sanduiche realizado no Instituto de Tecnologia Química Biológica da Universidade Nova de Lisboa (Portugal), é descrito o uso do sistema de monohíbrido em leveduras visando a identificaçao de fatores de transcrição capazes de se ligar ao promotor de *OsAPX8.* Entre os fatores de transcrição identificados, verificamos potenciais ativadores da expressão gênica, que são fortemente regulados pelo estresse hídrico, e que, portanto, podem contribuir com o entendimento dos mecanismos que envolvem a regulação de *OsAPX8* em plantas submetidas ao estresse. Desta forma, os dados apresentados neste capítulo, apesar de preliminares, podem permitir a caracterização de novas vias de transdução de sinais que medeiam a resposta a seca, via modulação da expressão de OsAPX8.

INTRODUÇÃO

A seca é um dos principais estresses abióticos que afetam o desenvolvimento vegetal e limitam a produção de sementes. O controle da perda de água pelas plantas se dá principalmente pela atividade dos estômatos, que são poros localizados na epiderme das folhas e que controlam a captação de CO_2 para os processos fotossintéticos e a perda de água durante a transpiração (SCHROEDER *et al*., 2001; HETHERINGTON & WOODWARD, 2003). Durante o estresse de seca, o fechamento dos estômatos é mediado principalmente por ácido abscísico (ABA) em um mecanismo que permite a adaptação à falta de água (SCHROEDER *et al*., 2001). Muitos trabalhos já demonstraram que diferentes espécies reativas de oxigênino (ROS, do inglês, *reactive oxygen species*) incluindo peróxido de hidrogênio (H_2O_2), gerados em condições de estresse, podem atuar como segundo mensageiros na sinalização por ABA (MCAINSH *et al*., 1996; PEI *et al*., 2000; KOHLER *et al*., 2003; BRIGHT *et al*., 2006), sendo capazes de promover o fechamento estomático (MCAINSH *et al*., 1996; ZHANG *et al*., 2001). No entanto, os genes que regulam o aumento de H_2O_2 em resposta ao estresse ainda não foram totalmente identificados, e os mecanismos de modulação dos níveis de H_2O_2, especialmente em estômatos, é em grande parte desconhecido.

Em arroz (*Oryza sativa*), a APX é codificada por uma família gênica composta de oito genes, denominados de *OsAPX1* a *OsAPX8* e as suas diferentes isoformas apresentam padrões distintos de expressão, em decorrência do estímulo ambiental. Ao contrário das demais formas de APX, em situações de estresses abióticos, como alta luminosidase, frio e seca, a isoforma cloroplastídica *OsAPX8* possui sua expressão modulada negativamente (MENEZES-BENAVENTE *et al*., 2004; TEIXEIRA *et al*., 2004; 2005; ROSA *et al*., 2010; BONIFACIO *et al*., 2011, CAVERZAN *et al*., 2014). Além disso, conforme demonstrado nos capítulos anteriores, a análise de plantas de arroz silenciadas por RNAi nas isoformas cloroplastídicas de APX (*OsAPX7* e *OsAPX8*), demonstram que o silenciamento de *OsAPX8* induz mudanças na

geração de *ROS* e no fechamento estomático. Estes dados indicam uma possível participação de *OsAPX8* nos mecanismos de fechamento estomático em resposta ao déficit hídrico.

Em arroz, um fator de transcrição do tipo “zinc-finger”, DST (do inglês, *drought and salt tolerance*), foi descrito como um regulador da abertura estomática em resposta a seca e salinidade (HUANG *et al*., 2009). DST contribui para a modulação da abertura estomática via regulação de genes envolvidos na homeostase de *ROS* e sua expressão é modulada negativamente por ABA e por estresse de seca, induzindo assim um aumento da geração de H_2O_2 (LI *et al*., 2013). Análises *in silico* revelaram a presença de pelo menos três possíveis sítios de ligação ao DST na região promotora de *OsAPX8*. Esses sítios de ligação foram denominados *DBS* (do inglês, *DST bindiing site*). Esses dados sugerem que o fator de transcrição DST possa regular a expressão de OsAPX8, que participaria dos mecanismos de resposta ao estresse de seca através da modulação dos níveis de *ROS* no cloroplasto.

OBJETIVOS

Dada a modulação da expressão de *OsAPX8* em resposta a diferentes estresses, e sua possível participação nos mecanismos de fechamento estomático, neste trabalho se objetivou a identificação de fatores de transcrição capazes de regular a expressão de *OsAPX8*. Duas abordagens foram utilizadas:

- Avaliar a capacidade do fator de transcrição DST em se ligar ao promotor de *OsAPX8*;
- Identificar outros fatores de transcrição capazes de se ligar ao promotor de *OsAPX8*;

MÉTODOS

Identificação de fatores de transcrição utilizando a técnica de mono híbrido em levedura (yeast one hybrid).

Os fatores de transcrição capazes de se ligar a região promotora de *OsAPX8* foram identificados através da triagem de bibliotecas de cDNA de expressão obtidas de plantas submetidas a estresses abióticos, como alta salinidade, seca ou frio (bibliotecas disponíveis no Laboratório de Genômica de Plantas em Estresse, do Instituto de Tecnologia Química e Biológica da Universidade Nova de Lisboa, Oeiras, Portugal), utilizando o sistema de monohíbrido em levedura (Y1H). Para tal foram construídas três estirpes de levedura (estirpes isca), contendo fragmentos do promotor (até 1500 pb antes do ATG). Desta forma, foram obtidas três construções diferentes: fragmento A (sendo este o mais próximo ao ATG) com aproximadamente 400 pb; fragmento B com aproximadamente 500 pb; fragmento C com aproximadamente 400 pb (Figura 1).

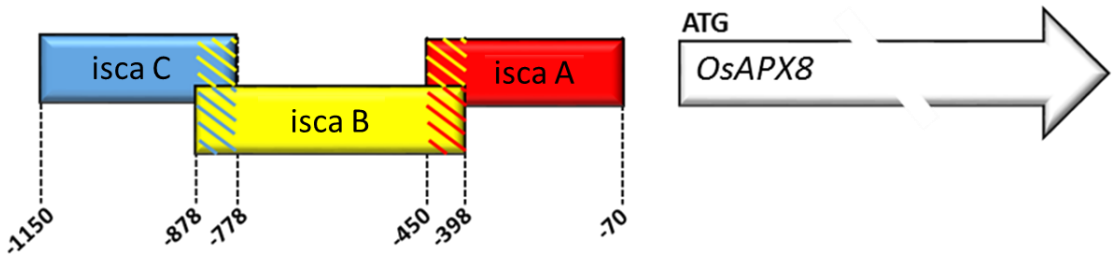

Figura 1. Esquema dos fragmentos da sequência promotora do gene da *OsAPX8* usados como isca nos experimentos de monohíbrido. As iscas foram construídas a partir de fragmentos sobrepostos da sequência promotora do gene e estas foram denominadas de Isca A (vermelho); Isca B (amarelo) e Isca C (azul).

Cada fragmento (denominados de isca A, isca B e isca C) foi clonado em vetor pHISi e integrado no genoma da cepa de levedura Y187 (Clontech). Cada linhagem foi repetidamente re-transformada com duas bibliotecas de cDNA (biblioteca de plantas submetidas a estresse de seca e biblioteca de plantas submetidas a estresse de frio; FIGUEIREDO *et al*., 2012), e crescida em meio seletivo com a concentração de 3AT (inibidor da síntese endógenase de histidina) determinada previamente (isca A: SD-His + 40mM 3AT, isca B: SD-His + 20mM 3AT e isca C: SD-His + 20mM 3AT). Dessa forma, as leveduras que não estiverem expressando o gene *HIS* (que está sob o constrole dos fragmentos do promotor de *OsAPX8*) não será capaz de crescer na ausência de histidina exógena, a não ser que através do "screening" com a biblioteca a levedura receba um clone contendo um cDNA que codifique uma proteína capaz de se ligar aos fragmentos do promotor. As sequencias de cDNA da biblioteca estão clonadas em vetor pACTII, que codifica um domínio de ativação em fusão com a proteína da biblioteca, capaz de induzir a expressão de *HIS* caso a proteína codificada pelo cDNA selecionado por meio do "screening" se ligue aos fragmentos do promotor de *OsAPX8*. Para cada fragmento do promotor foram triados mais de um milhão de clones de cada biblioteca. As colônias de levedura que conseguiram crescer no meio seletivo foram analisadas por PCR para verificação quanto à presença do plasmídeo da biblioteca utilizada e os produtos de PCR foram sequenciados e analisados por BLAST para a identificação do cDNA codificante. A validação destas interações foi realizada por re-transformação com o plasmídeo purificado.

Avaliação da interação entre o fator de transcrição DST e o promotor de *OsAPX8* utilizando a técnica de Yeast One Hybrid

As linhagens de leveduras contendo as regiões do promotor de *OsAPX8* foram co-transformadas com o vetor pDEST 22 contendo o cDNA codificante do fator de transcrição DST clonado em fusão com um domínio de ativação da transcrição da proteína GAL4. As leveduras foram selecionadas em meio SD sem histidina, objetivando analisar a capacidade do fator de transcrição DST em se ligar ao promotor de *OsAPX8*.

RESULTADOS E DISCUSSÃO

Avaliação da interação entre o fator de transcrição DST e o promotor de *OsAPX8*

Após a obtenção das linhagens de leveduras contendo os fragmentos do promotor de *OsAPX8*, estas foram transformadas com o vetor pDEST22 contendo a sequencia codificante do fator de transcrição DST. Os resultados demonstraram uma forte interação entre o DST e a isca A do promotor de *OsAPX8* (Figura 1). Não foram identificadas, no entanto, interações entre DST e as iscas B e C.

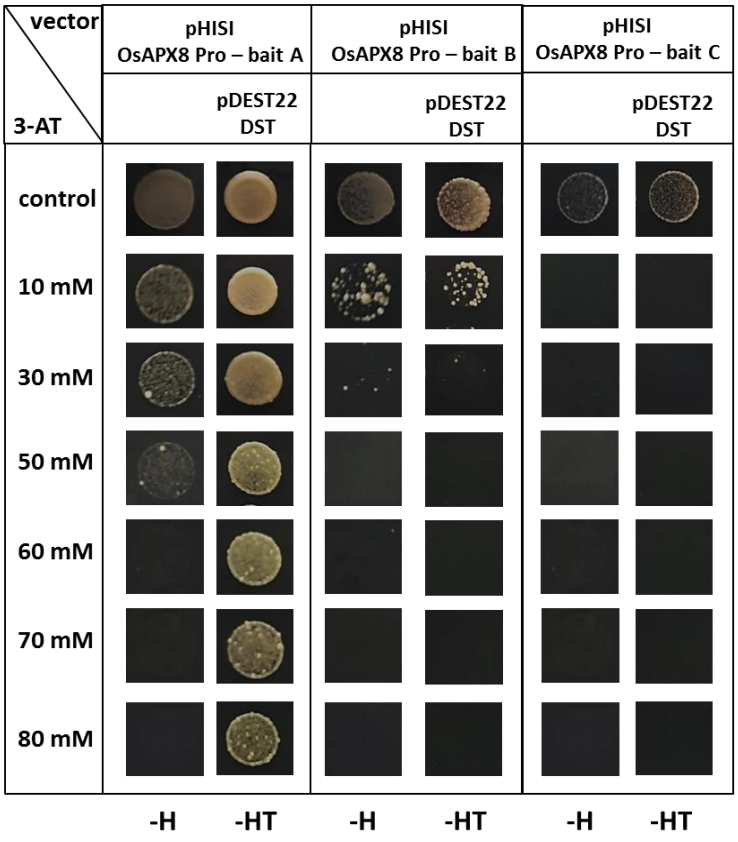

Figura 1. Avaliação da interação entre o fator de transcrição DST e os fragmentos do promotor de OsAPX8 por monohíbrido em levedura. **Foram utilizadas diferentes concentrações de 3-AT, afim de se determinar a concentração mínima capaz de inibir a sintese endógena de histida, proveniente de auto-ativação.**

Dados da literatura, indicam que o fator de transcrição DST contribui para a regulação da abertura estomática via regulação de genes envolvidos na homeostase redox, como peroxidases (precursor 24, precursor 12), glutatione S-transferase (Os*GSTU2*) e citocromos da família P450 (71D10, 94A2). Sob condições de estresses de seca e salinidade a expressão de DST é fortemente inibida, o que, indiretamente, levaria ao acúmulo de *ROS* (HUANG *et al*., 2009). De fato, o gene de Os*APX8* também é reprimido durante o estresse de seca e o silenciamento deste gene leva ao aumento do acúmulo de *ROS* e do fechamento estomático, conforme demonstrado no capítulo 4. Portanto, a ligação desse fator de transcrição no promotor do gene *OsAPX8* e o padrão coincidente da expressão de ambos em resposta a seca indicam que os mesmos participam dos mecanismos que culminam com o fechamento dos estômatos na resposta ao estresse por falta de água.

Identificação de fatores de transcrição capazes de se ligar ao promotor de *OsAPX8*

O "*screening"* da biblioteca de cDNA obtida de plantas submetidas a estresse de seca não resultou na identificação de fatores de transcrição com capacidade de se ligar à região promotora de *OsAPX8*. Mesmo após a triagem de mais de um milhão de clones para cada fragmento isca, nunhum clone positivo foi identificado. Devido a isso, novos experimentos foram realizados visando o "screening" de biblioteca de cDNA de plantas submetidas a frio, tendo sido triados pelo menos um milhão de clones para cada fragmento isca do promotor de *OsAPX8*. Utilizando esta segunda biblioteca foram identificados três fatores de transcrição com capacidade de se ligar ao promotor de *OsAPX8*. Os fatores de transcrição identificados estão listados da tabela 1. A confirmação destas interações foi realizada através da re-transformação das estirpes isca com o plasmídeo purificado (vetor pACTII) (Figura 2).

Tabela 1. Genes que codificam as proteínas capazes de se ligar à região promotora de *OsAPX8* identificados a partir do "screening" de bibliotecas de cDNA pelo sistema de monohíbrido em levedura.

Nome do gene	Família	Locus	Isca utilizada
Os11g0472000	C3H	LOC_Os11g28270.1	Isca A
OJ1119_H02.18	bZIP	LOC_Os05g41070.1	Isca A
OsVOZ1	VOZ	LOC_Os01g54930.1	Isca B

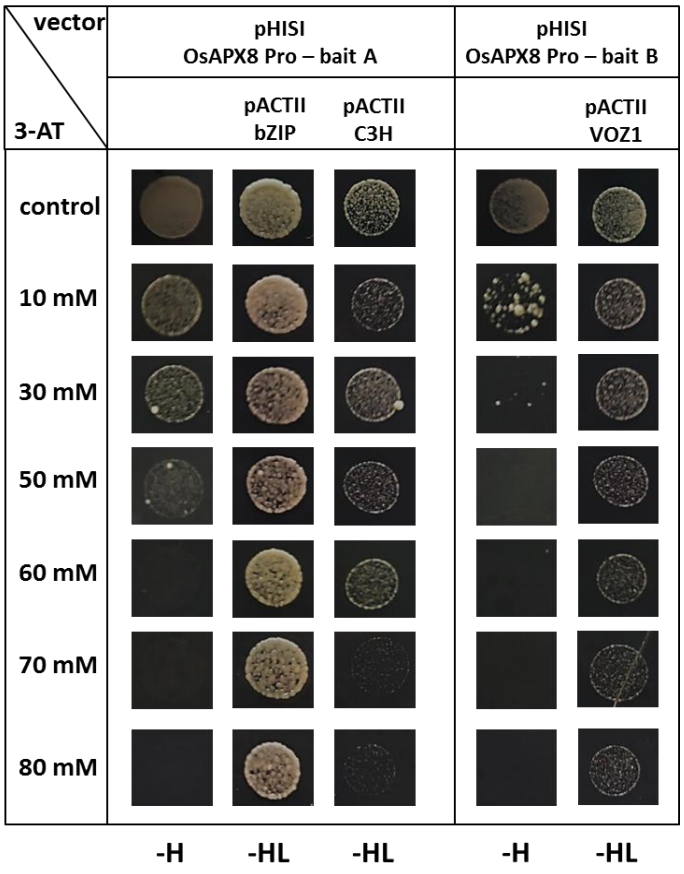

Figura 2. Confirmação dos fatores de transcrição capazes de se ligar ao promotor de *OsAPX8* por monohíbrido em levedura.

Um dos fatores de trancrição identificados, corresponde a uma proteína do tipo “dedo de zinco” e é codificado pelo gene *Os11g28270.* No entanto, informações a respeito desta proteína em arroz não estão disponíveis. Já em *Arabidopsis thaliana,* o homologo ZFN1 (ZINC FINGER PROTEIN 1; *AT3G02830*), também conhecido como PENTA 1), atua como um inibidor da expressão gênica e está possivelmente envolvido no desenvolvimento da parte aérea da planta, bem como na ativação da fotomorfogênese (CHRISPEELS et al., 2000). De fato, em um trabalho anterior foi demonstrado que ZFN1 é capaz de interagir com fitocromo em resposta a luz vermelho-distante, e regular negativamente a transcrição de protoclorofila reductase (PORA), responsável pela conversão de protoclorofila em clorofila (PAIK et al., 2012).

Outro fator de transcrição capaz de se ligar ao promotor de Os*APX8*, foi a proteína OsbZIP42 ou OsABF7 (*ABA-responsive element binding factor 7*), que atua como ativador da expressão gênica, e cuja expressão é reprimida em resposta ao tratamento com PEG (capaz de simular o estresse hídrico), NaCl e fitohormônios, como metil-jasmonato e NAA (Ácido 1-naftalenoacético) (LU et al., 2009). A família de fatores de transcrição do tipo ABF/AREB é capaz de se ligar ao DNA através do elemento de resposta ao ABA (ABRE, do inglês *abscisic acid response element*), e a análise do promotor de *OsAPX8*, através do banco de dados PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), demonstra que a sequência referente a este cis-elemento (ACCACGTGTC) está presente entre as iscas A e B (posição -440 em relação ao ATG). Em *Arabidopsis thaliana*, esta proteína possui alta homologia com o fator de transcrição AtbZIP66 ou AtAREB3 (ABA-responsive element binding protein 3;*AT3G56850*), expresso no embrião durante a maturação da semente (BENSMIHEN et al., 2005), mas não verificado em plantas adultas, mesmo sob condições de estresse (UNO et al., 2000).

O último fator de transcrição identificado corresponde a proteína OsVOZ1 (*vascular plant one-zinc-finger protein*), um possível ativador da expressão gênica. Esta proteína faz parte da família de fatores de transcrição do tipo VOZ, que é composta por dois membros, VOZ1 e VOZ2, presentes em monodicotiledôneas e eudicotiledôneas. Embora o papel fisiológico destas

proteínas em arroz ainda não tenha sido estudado, em *Arabidopsis thaliana*, os genes VOZ respondem a diferentes estresses abióticos, sendo fortemente reprimidos em plantas submetidas a seca e a baixas temperaturas. Além disso, plantas duplo mutantes para *AtVOZ1* e *AtVOZ2* (voz1/voz2) apresentaram um aumento da tolerância a esses estresses. Por outro lado, a resistência destas plantas, a patógenos como o fungo *Colletotrichum higginsianum* e a bactéria *Pseudomonas syringae* foi fortemente reduzida (NAKAI et al., 2013). Posteriormente, foi demonstrando que linhagens que superexpressam *AtVOZ1* e *AtVOZ2* apresentavam uma redução da tolerância a estresses abióticos (seca e frio), mas em relação a patógenos, elas apresentavam um aumento da resistência (NAKAI et al., 2013a). Estes dados, portanto, indicam que os fatores de transcrição do tipo VOZ, possuem um importante papel na resposta a estresses biótico e abióticos e poderiam regular as respostas mediadas por OsAPX8.

CONCLUSÕES E PERSPECTIVAS

Através de experimentos de monohíbrido em levedura, demonstramos que o fator de transcrição OsDST é capaz de se ligar ao promotor de *OsAPX8.* Além disso identificamos por “screening” de bibliotecas de cDNA, outros três fatores de transcrição capazes de se ligar ao promotor de Os*APX8*: LOC_Os11g28270, LOC_Os05g41070 (OsABF7) e LOC_Os01g54930 (*OsVOZ1*). Com base nos dados disponíveis na literatura concluímos que as proteínas OsDST, OsABF7 e OsVOZ1, são fortes candidatos a serem, de fato, membros de vias de transdução de sinais mediadas por *OsAPX8*. Em geral, todos estes fatores de transcrição são ativadores da expressão gênica, e são fortemente reprimidos durante o estresse de seca. Se de fato, tais proteínas forem capazes de ativar a expressão de *OsAPX8*, a repressão da expressão de cada fator de transcrição, em condições de estresse, poderia contribuir para a redução da expressão de *OsAPX8*, colaborando para o aumento da geração de *ROS* e do fechamento estomático nestas condições (Figura 3).

Como perspectiva deste trabalho, pretendemos avaliar, por ensaio de transativação em protoplastos de arroz, a capacidade dos fatores de transcrição identificados em ativar ou inibir a expressão gênica sob o controle do promotor de *OsAPX8*. Além disso, o próprio ensaio de transativação pode ser utilizado para confirmar as interaçõeso DNA-proteína, uma vez que, nos experimentos que utilizam o sistema de protoplastos de plantas, além das proteínas estarem completas, elas estarão em sua conformação correta e com as modificações pós-traducionais apropriadas, uma vez que estarão sendo expressas em células de arroz, e não em leveduras. Além disso, pretendemos avaliar a expressão dos fatores de transcrição identificados na resposta de plantas de arroz a diferentes estresses abióticos. Estes ensaios permitirão a caracterização de rotas de sinalização que modulem a expressão de *OsAPX8* em resposta ao estresse.

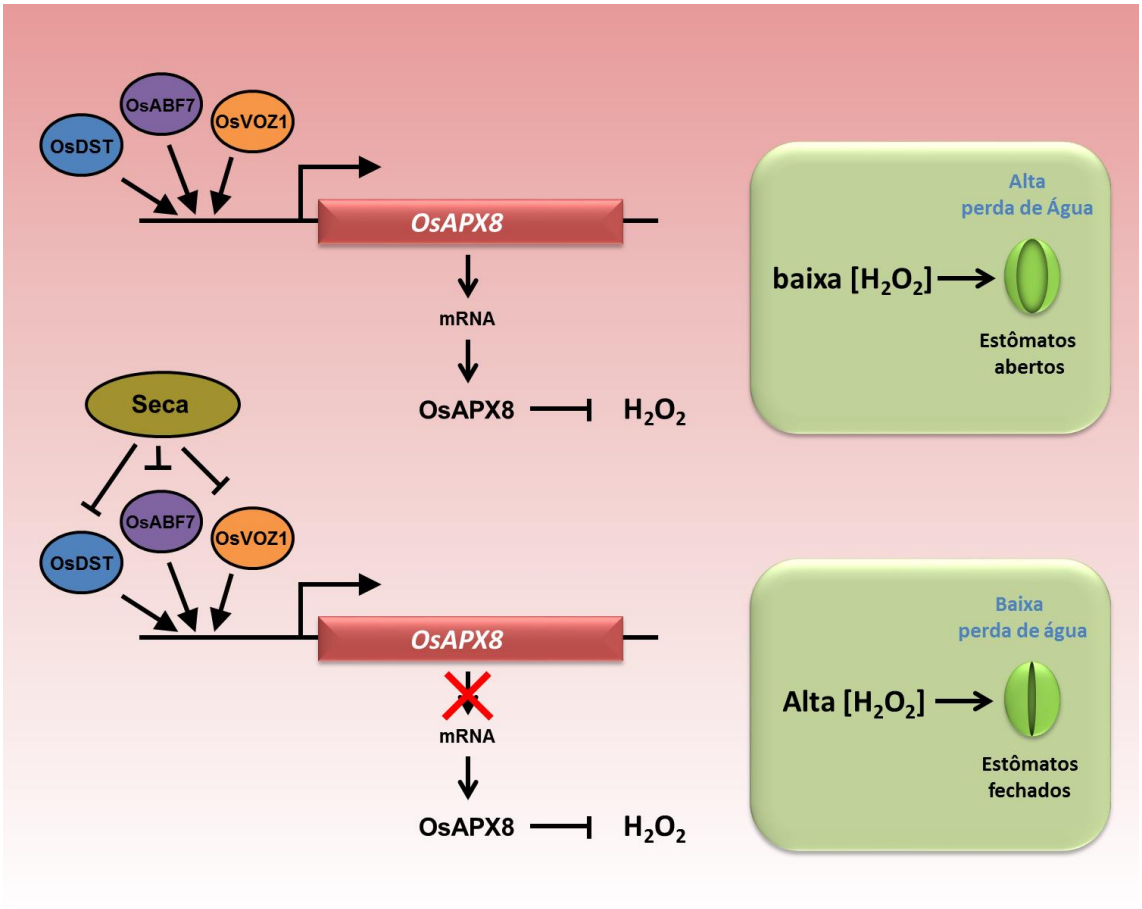

Figura 3. Modelo proposto para a regulação da expressão de OsAPX8 e seu papel no controle do fechamento estomático em resposta ao estresse de seca. *Os fatores de transcrição OsDST, OsABF7 e OsVOZ1 seriam capazes de se ligar diretamente no promotor de OsAPX8 e ativar a expressão gênica, inibindo, portanto, o acúmulo de ROS. Sob condição de estresse de seca, a expressão destes fatores de transcrição é reprimida, resultando na regulação negativa da expressão de OsAPX8, o que leva a acúmulo de ROS e o fechamento estomático*.

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4- DISCUSSÃO GERAL

As *ROS* são formadas como subproduto do metabolismo aeróbico em diferentes organismos e atuam como moléculas sinalizadoras, ou mensageiros secundários, em uma complexa rede de sinalização em diferentes organismos. Desta forma, em plantas, as vias de produção de ROS, bem como os mecanismos antioxidantes são fundamentais para o desenvolvimento vegetal, e para a resposta a diferente estímulos ambientais.

Neste trabalho, identificamos uma nova e importante fonte de geração de ROS em células vegetais: a enzima SDH, que corresponde ao complexo II da respiração. Os resultados obtidos demonstram que a geração de ROS pela SDH pode ser modulada por diferentes inibidores. Enquanto inibidores não-competitivos, como ácido salicílico e óxido nítrico (NO), são capazes de induzir a geração de *ROS*, os inibidores competitivos são capazes de previnir a geração de *ROS* pela SDH. Este mecanismo se mostra importante em células vegetais, pois diferentes moléculas endógenas podem atuar regulando a geração de *ROS* pela SDH. Além disso, este mecanismo resulta na regulação da expressão de genes relacionados com o desenvolvimento vegetal e com a tolerância a diferentes estresses.

Se por um lado a manipulação da geração de *ROS* pela mitocôndria promoveu alterações claras no padrão de desenvolvimento, alterações nas respostas antioxidantes no cloroplasto afetaram em menor grau o crescimento da planta. No entanto, impactos nos mecanismos de defesa foram verificados em resposta às alterações em ambas organelas, de maneira similar. Assim, nossos dados demonstram que as isoformas cloroplastídicas de APX também estão envolvidas em mecanismos de tolerância ao estresse, indicando o potencial para um papel na regulação dos níveis de *ROS* por enzimas relacionadas com a produção e a eliminação dessas moléculas reativas. No entanto, apesar desses pontos comuns, a complexidade das respostas obtidas indica uma alta especificidade das respostas, que depende da localização subcelular de cada um dos componentes dessa intricada rede, assim como o

nível de expressão de cada um deles. Por exemplo, enquanto o silenciamento duplo de *OsAPX7* e *OsAPX8*, bem como o silenciamento individual de *OsAPX8,* resultou em plantas com um maior conteúdo de *ROS* e maior fechamento estomático em codições ideais, o silenciamento individual de *OsAPX7* não resulta em alterações fisiológicas nas plantas silenciadas. No entanto, as plantas silenciadas para OsAPX7 e as plantas duplamente silenciadas para ambos os genes demonstram uma maior tolerância ao estresse de seca. Esses dados demonstram que o aumento da tolerância à seca não é consequência somente do aumento de *ROS* ou do fechamento estomático, uma vez que tais fenômenos não são observados nas plantas silenciadas para *OsAPX7*.

O aumento da tolerância a seca também foi verificado em plantas nocaute para *OsAPX8* (*apx8/apx8*). Curiosamente, ao contrário das plantas silenciadas, as plantas nocaute pra *OsAPX8* não apresentaram mudanças fisiológicas em condições ideias, e dados de RNAseq demonstram que o silenciamento de *OsAPX8* induz uma maior alteração na expressão gênica do que o nocaute. Aparentemente o silenciamento de OsAPX8 simula uma condição de estresse, onde a expressão de *OsAPX8* seria reduzida naturalmente. Desta forma, as plantas silenciadas para *OsAPX8* apresentam um fenótipo parecido ao de plantas submetidas a estresse, como um maior conteúdo de ROS, maior fechamento estomático, crescimento mais lento, bem como uma maior resposta na expressão gênica. Por outro lado, o nocaute de *OsAPX8* estaria gerando compensações não verificadas nas plantas silenciadas, que permitem as plantas nocaute apresentar um fenótipo mais parecido com plantas NT, embora sejam mais tolerantes ao estresse de defict hídrico. No entanto, os mecanismos capazes de induzir o aumento da tolerância dessas plantas ao estresse de seca ainda são desconhecidos.

Além das avaliações de interações gênicas e efeitos de manipulações genéticas, a análise de como esses componentes envoluíram nos diferentes genomas pode trazer novos dados para o entendimento da função de cada um deles nos mecanismos de defesa das plantas. Nesse sentido, o estudo da história evolutiva da succinato desidrogenase, descrita no capítulo 2 indicou que esta enzima é membro da superfamília de fumarato redutases, composta por

outras enzimas, como a fumarato redutase dependente de quinona, NADH ou thiol, além da enzima L-aspartato redudase. Nossos dados demonstram que todas essas enzimas se originaram de um acestral comum, e os processos de endossimbiose da mitocôndria e do cloroplasto assumiram um papel central na distribuição destas enzimas em organismos eucariotos.

Nesta tese, demonstramos que os diferentes mecanismos envolvidos no balanço redox de células vegetais exercem um importante papel no desenvolvimento vegetal e na reposta de plantas a diferentes estresses. Além disso, identificamos diferentes fatores de transcrição capazes de se ligar ao promotor de *OsAPX8*, o que indiretamente também poderia regular o balanço redox celular. A avaliação da capacidade dessas proteínas em regular a expressão gênica permitirá ainda a indentificação de novas vias de sinalização envolvidas na resposta ao estresse. Embora ainda existam incontáveis aspectos a serem explorados nesta área, o presente trabalho contribuiu para a identificação de novas interações e, consequentemente de vias inéditas de sinalização nas células vegetais, ampliando a visão do papel das *ROS* nos mecanismos de respostas a estímulos ambientais geradores de estresse oxidativo.

5- CONCLUSÕES GERAIS

- Succinato desidrogenase é um importante sítio de geração de ROS em células vegetais e regula a expressão de genes envolvidos com o desenvolvimento vegetal e a resposta ao estresse;

- A família de succinato:quinona oxiredutase é membro de uma superfamília de fumarato redutases, que se originou apartir de um ancestral comum e está envolvida em processos metabólicos chave ao longo da evolução das espécies;

- As isoformas cloroplastídicas de ascorbato peroxidase desempenham um importante papel na homestase redox e no controle da abertura estomática e da tolerância ao estresse hídrico em arroz;

- Os fatores de trancrição, OsDST, OsVOZ1, OsABF7 e LOC_Os01g54930 são capazes de se ligar ao promotor de *OsAPX8* e são potenciais reguladores da expressão gênica;

- As *ROS* são essenciais no controle das respostas das plantas a diferentes estresses.

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