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Excesso de ferro em arroz (*Oryza sativa* L.): efeitos tóxicos e mecanismos de tolerância em distintos genótipos

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Ne pas posséder, être.

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Resumo

O ferro é um elemento essencial para o crescimento e desenvolvimento das plantas, envolvido em processos metabólicos essenciais, como fotossíntese e respiração. Entretanto, quando livre e em excesso, pode gerar estresse oxidativo. A toxidez por excesso de ferro trata-se do maior problema nutricional em arroz alagado, sendo responsável por perdas na produtividade. Diversas estratégias para minimizar os efeitos tóxicos do ferro vêm sendo desenvolvidas, e entre elas, o uso de cultivares tolerantes é considerada a mais efetiva. Porém, poucos dados com relação à interação entre diferentes genótipos de arroz e o ambiente encontram-se disponíveis. Utilizando-se de abordagens bioquímicas e moleculares, foram analisadas as respostas de diferentes cultivares de arroz expostas a altos níveis de ferro, crescidas em campo ou em laboratório. A toxidez por excesso de ferro teve um claro efeito foto-oxidativo, levando a quedas nos teores de clorofila, bem como a danos oxidativos. Excessivos níveis de ferro levaram a um aumento na atividade de enzimas antioxidantes, bem como a alterações no estado oxidativo da célula, modificando as concentrações das formas oxidadas e reduzidas de ascorbato e glutationa. A concentração de ferro apresentou-se variável nas cultivares tolerantes testadas. Os dados obtidos indicam que possíveis mecanismos de tolerância ao excesso de ferro podem envolver a capacidade de acumular ferro em frações superiores a 3kDa, maior atividade de SOD (através da expressão diferencial de três isoformas), bem como a limitação da captura do metal, possivelmente envolvendo a lignificação e remodelamento da parede celular das células da raiz. Altos níveis de ferro levaram ao acúmulo de transcritos dos genes de ferritina, em especial de *OsFER2*, dependente de um passo oxidativo, bem como à expressão de outros genes relacionados a homeostase de ferro. Cinco genes pertencentes às famílias gênicas ZIP (*OsZIP1*, *OsZIP7* e *OsZIP8*) e NRAMP (*OsNRAMP4* e *OsNRAMP5*) tiveram sua expressão

induzida em plantas expostas a altos níveis de ferro, sugerindo seu possível envolvimento em respostas ao excesso de ferro. Os genes *OsIRT1* e *OsIRT2*, *OsNRAMP1* e *OsYS7*, cuja expressão relativa foi aumentada em condições de deficiência de ferro, tiveram sua expressão reduzida em excesso de ferro. Esses genes codificam transportadores de alta afinidade por ferro, sugerindo a ocorrência de uma resposta coordenada, dependente da concentração de ferro. Plantas de cultivares de arroz distintas apresentaram diferentes mecanismos de tolerância ao excesso de ferro.

Palavras-chave: arroz, enzimas antioxidantes, excesso de ferro, ferritina, ferro.

Abstract

Iron is an essential nutrient for growth and development of plants, involved in important plant biological processes, such as photosynthesis and respiration. However, when free and in excessive levels inside the cell, iron can act as a pro-oxidant, leading to oxidative stress. Iron toxicity is considered the major nutritional disorder in waterlogged and lowland rice, being responsible for losses on rice production. Several management strategies have been developed to overcome iron toxicity, and the most cost-effective approach is the use of tolerant rice cultivars. Despite this, few data concerning the relation between different rice cultivars and its environment are available. Through the use of molecular and biochemical approaches, we analyzed the responses of distinct rice genotypes exposed to iron excess, cultivated in the field or in the laboratory. Iron toxicity had a clear photo oxidative damage, leading to decreases in chlorophyll levels and generating oxidative damage. Iron excess also induced the activity of antioxidant enzymes, as well as an alteration in the redox status of the cell, besides concentration varied between the studied cultivars. Mechanisms involved in the tolerance to iron toxicity may involve the capacity to accumulate iron at molecular mass fractions, a higher SOD activity (probably through the differential induction of SOD isoforms), and also the limitation of iron uptake in nutrient solution. This limitation may rely in the root cell wall remodeling and lignifications. Iron lead to an up-regulation of ferritin genes, especially *OsFER2*, with this induction being dependent on an oxidative step. Iron excess also lead to an induction on the relative gene expression of iron homeostasis-related genes. Five genes belonging to two distinct gene families, ZIP (*OsZIP1*, *OsZIP7* and *OsZIP8*) and NRAMP (*OsNRAMP4* e *OsNRAMP5*), were up-regulated in plants exposed to iron excess, suggesting their possible role in response to excessive amounts of iron. Interestingly, five genes (*OsIRT1* and

OsIRT2, *OsNRAMP1* and *OsYS7*) up-regulated by iron deficiency, were regulated in an opposite way by iron excess. All the five genes encode proteins involved in the uptake and transport of iron, suggesting a coordinated response, depending on the iron concentration. Taken together, our results indicated that different rice cultivars can use distinct tolerance mechanisms.

Key words: antioxidative enzymes, ferritin, Fe, iron toxicity, rice.

Lista de Abreviaturas

A/Ci= CO2 assmilation rate/ estimated substomatal CO2 partial preassure
A= CO2 assimilation rate
AA= ascorbic acid
ABA= abscisic acid
ANOVA= analysis of variance
APX= ascorbate peroxidase
ASR= absicisic acid-stress-reapining
AtFer1= Arabidopsis thalina ferritin 1
BAP= benzyl aminopurine
BSA= bovine serum albumin
C.E.C= cation exchange capacity
CAT= catalase
cDNA= complementar deoxynucleic acid
CONAB= Companhia Nacional do Abastecimeno
Ct= threshold cycle
Ctrl= control
Cu/ZnSOD= Cu/Zn superoxide dismutase
Cytb6/f= cytochrome b6/f
DCB= dithionite-citrate-bicarbonate
DHAR= dehydroascorbate reductase
DNA= deoxynucleic acid
DW= dry weight
E= amplification efficiency
EDTA= ethylenediaminetetracetic acid
FeSOD= Fe superoxide dismutase
G-POX= guaiacol peroxidase
GR= glutathione reductase
GSH= glutathione
GSSG= glutathione disulfide
HEPES= 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IDRS= iron dependent regulatory sequence
IRE= Iron Regulatory Elements
IRGA= Instituto Riograndense do Arroz
IRT= iron regulated transporter
ITP= iron transport protein
JCR= journal of citation reports
Jmax= electron transport rate
LHC II= light harvesting complex II
MES= 2-(N-morpholineethanesulfonic acid)
MnSOD= Mn superoxide dismutase
mRNA= messenger ribonucleic acid
NA= nicotianamine
NAC= NAM, ATAF and CUC
NADPH= nicotianamide adenine dinucleotide phosphate

NO= nitric oxide
NRAMP= Natural resistance associated macrophage protein
O.M.= organic matter
OsAct= *Oryza sativa* actin
OsFER= *Oryza sativa* ferritin
OsFRDL1= *Oryza sativa* frd-like 1
OsIRO2= *Oryza sativa* open reading frame 2
OsSgr= *Oryza sativa* staygreen
OsUbq= *Oryza sativa* ubiquitin
OsYS= *Oryza sativa* yellow stripe
PCR= polymerase chain reaction
PMSF= phenylmethylsulphonylfluoride
PPF= photosynthetic photon flux
PSII= photosystem II
PVP= polyvinyl pyrrolidone
QTL= quantitative trait loci
RMA= robust multichip analyses
RNA= ribonucleic acid
ROS= reactive oxygen species
RT-PCR= reverse transcribed polymerase chain reaction
siRNA= small interference RNA
SNP= sodium nitroprusside
SOD= superoxide dismutase;
TBARS= thiobarbituric reactive species
TCA= tricloroacetic acid
TIGR= The Institute for Genomic Research
V_{cmax}= maximum carboxilation rate
YS= yellow stripe
ZIP= ZRT/IRT-related transporters

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Introdução

O Rio Grande do Sul ocupa uma posição de destaque na produção de arroz no Brasil. Segundo dados da Companhia Nacional do Abastecimento (CONAB), na safra de 2006/2007 a produção de arroz no estado correspondeu a 65 % da produção brasileira. Na safra de 2006/2007 foram plantados 1.144,2 hectares da cultura, sendo colhidas 7.282,4 toneladas de grãos. A principal, e mais produtiva forma de cultivo de arroz no estado é o sistema de alagamento (Instituto Rio Grandense do Arroz).

Em solos ácidos, a anoxia e redução do pH, decorrentes do alagamento, podem levar à solubilização de grandes quantidades de ferro, antes precipitado formando quelatos e ligado à matéria orgânica presente no solo (Ponnamperuma, 1972). Solos alagados são submetidos a mudanças periódicas entre condições aeróbicas e anaeróbicas. Como o oxigênio difunde-se mais rapidamente no ar do que na água (Armstrong, 1979), é rapidamente utilizado por microorganismos e raízes de plantas. Em ambientes alagados, após a depleção do oxigênio, outros íons são utilizados como aceptores de elétrons (e.g. NO_3^-, Mn^{4+}, Fe^{3+} e SO_4^{-2}) por microrganismos anaeróbicos facultativos, sendo subsequentemente reduzidos (Ponnamperuma, 1972). A concentração de ferro solúvel, que anterior à submergência do solo raramente excede 0,1 mg L^{-1}, pode chegar, em solos ácidos, a aproximadamente 600 mg L^{-1} (Ponnamperuma *et al.*, 1978). Entretanto, em casos extremos, já foram detectados valores de até 5.000 mg L^{-1} (Hansen & van Breemen, 1975).

Os principais sintomas de toxidez por excesso de ferro são o bronzeamento das folhas, inicialmente as mais velhas, e deposição de pigmentos marrons, podendo levar ao retardo no crescimento, baixa produtividade, esterilidade das espiguetas, e em casos mais severos, morte da planta (Ponnamperuma *et al.*, 1955). Perdas na produção de arroz

decorrentes da toxidez por excesso de ferro podem levar a prejuízos de 15 a 20 %; entretanto, perdas totais na produção já foram descritas na literatura (Audebert & Sahrawat, 2000; Winslow *et al.*, 1989).

O ferro, quando livre e em excesso dentro da célula, é capaz de gerar radicais livres, como o radical hidroxila, através da reação de Fenton (Becana *et al.*, 1998). Este radical é extremamente tóxico para o metabolismo celular, sendo responsável pela oxidação de macromoléculas biológicas como proteínas, ácidos nucléicos e lipídios de membrana (Halliwell & Gutteridge, 1984). A regeneração do átomo de íon ferroso pode ocorrer através da reação de Haber-Weiss com a redução do íon férrico a partir do ânion superóxido, tornando a produção de radicais hidroxilas um processo cíclico (Floyd, 1983). Em folhas destacadas de arroz submetidas a níveis tóxicos de ferro, altos níveis de peroxidação lipídica foram detectados, indicando oxidação de lipídeos causada por radicais livres, gerados através do acúmulo celular de ferro (Fang *et al.*, 2001), bem como aumento na atividade de peroxidases, envolvidas na detoxificação de peróxido de hidrogênio (Fang & Kao, 2000). Em estacas de *Nicotiana plumbaginifolia* cultivadas em sistema de hidroponia, o excesso de ferro levou a quedas de 40% na atividade fotossintética, queda de 30% no conteúdo celular de ascorbato e glutationa (dois agentes antioxidantes), assim como uma maior atividade de ascorbato peroxidase (Kampfenkel *et al.*, 1995). Logo, os níveis celulares de ferro devem ser finamente regulados para impedir danos celulares provocados por radicais livres.

Vários estudos identificando os principais efeitos da toxidez por excesso de ferro em arroz têm sido realizados, sendo identificados dois distintos tipos de toxidez por excesso de ferro: uma toxidez real (ou verdadeira) – caracterizada pelo acúmulo de grandes níveis de ferro em tecidos vegetais (Silveira *et al.*, 2007; Sahrawat, 2000; Olaleye *et al.*, 2001) e uma

toxidez indireta (ou pseudo) – caracterizada por uma desordem nutricional múltipla, causada pela deficiência de nutrientes essenciais como o K, P, Ca e Mg (Ottow *et al.*, 1983; Yamauchi, 1989). A ocorrência de toxidez indireta pode ser uma condição causada pelas grandes concentrações de ferro solúvel encontrado na solução líquida do solo ou uma conseqüência dos altos níveis de ferro no metabolismo da planta. Como indicado por Sahrawat (2004), o manejo destes dois distintos tipos de toxidez por ferro requer igualmente diferentes estratégias.

Cultivares de arroz com diferentes níveis de tolerância à toxidez por excesso de ferro foram desenvolvidas através de melhoramento genético (Fageria & Rabelo, 1987; Sahrawat *et al.,* 1996), e práticas agronômicas como plantio alternativo, manejo hídrico adequado, e a aplicação de fertilizantes (Benckiser *et al.,* 1984; Winslow *et al.,* 1989) vem sendo desenvolvidas e utilizadas. Neste panorama, a prática mais eficiente tem sido o uso de genótipos resistentes (Sahrawat *et al.,* 1996). Entretanto, devido à diversidade de ambientes em que a toxidez por excesso de ferro pode ocorrer, nenhuma destas opções é universalmente aplicável ou eficiente (Becker & Asch, 2005). Assim, o conhecimento sobre o impacto do excesso de ferro na fisiologia de plantas de arroz se torna necessário para a cultura.

À parte do significativo progresso no conhecimento das condições que levam à ocorrência da toxidez por excesso de ferro em arroz, ainda são pouco conhecidas as interações entre excesso de ferro e diferentes genótipos de arroz (Sahrawat, 2004). Diversos fatores envolvendo a tolerância ao excesso de ferro vêm sendo identificados, bem como a identificação de QTLs (*quantitative trait loci*) em distintas populações (Wu *et al.,* 1997; Wu *et al.,* 1998; Wan *et al.,* 2003; Shimizu *et al.,* 2005). Dentre os identificados na literatura, um QTL localizado na região C955-C885 do cromossomo 1 parece ter um

interesse particular, respondendo por 32,3% de variação na taxa de crescimento relativo de plantas expostas a altas concentrações de ferro (Wu *et al.,* 1998). O mesmo QTL foi identificado como responsável por 20,5% da variação no índice de bronzeamento foliar – um marcador visual de susceptibilidade ao excesso de ferro (Wan *et al.,* 2003).

Dentre possíveis mecanismos utilizados por diferentes cultivares para tolerar altos níveis de ferro, o envolvimento da capacidade oxidativa da raiz, levando à oxidação do ferro na superfície da raiz e formando uma "placa férrica", vem sendo sugerido como um mecanismo envolvido na resistência ao excesso de ferro (Ando *et al.,* 1983; Green & Etherrington, 1977). Apesar de classificado como um suposto mecanismo de tolerância ao excesso de ferro, o acúmulo de hidróxidos de ferro nas raízes tem um papel controverso. As propriedades químicas e físicas da placa férrica são similares aos óxidos de ferro encontrados no solo (Bacha & Hosnerr, 1977), sendo capaz de adsorver nutrientes, especialmente o P (Kuo, 1986). Assim, grandes quantidades de ferro precipitado nas raízes poderiam influenciar a absorção de nutrientes essenciais, possivelmente causando distúrbios nutricionais em plantas de arroz (Howeler, 1973; Armstrong & Armstrong, 1988).

Mecanismos de tolerância a altos níveis de ferro envolvendo a indução de sistemas antioxidantes (enzimáticos ou não) nas folhas de plantas de arroz têm sido igualmente sugeridos (Wu *et al.,* 1998; Yamauchi & Peng, 1995). Diversas enzimas e compostos antioxidantes participam do metabolismo de espécies reativas de oxigênio em plantas. Dentre as enzimas conhecidas, a desmutação de radicais superóxidos (O_2^-) é realizada pela enzima superóxido dismutase (SOD) (Rabinowitch & Fridovich, 1983), enquanto que a decomposição do peróxido de hidrogênio é mediada pelas enzimas da família ascorbato peroxidase (APX) (Asada, 1992), bem como pela enzima catalase (CAT) (Willekens *et al.*,

1997). A decomposição de peróxidos de lipídeos seria realizada por outras enzimas, como glutationa peroxidase (Beeor-Tzahar *et al.*, 1995), bem como a peroxiredoxina (Baier & Dietz, 1997), atuando como proteínas detoxificadoras de produtos secundários do estresse oxidativo (Mano, 2002).

Por outro lado, o ferro é um elemento essencial para o crescimento e desenvolvimento das plantas. É parte integrante do centro de oxirredução de inúmeras enzimas e moléculas de grande importância no metabolismo vegetal, como citocromos, nitrogenases e enzimas envolvidas na síntese da clorofila (Taiz & Zeiger, 1998).

O ferro é um elemento abundante na natureza, correspondendo a cerca de 5% da crosta terrestre. Contudo, grande parte encontra-se indisponível para as plantas, formando complexos insolúveis na presença de oxigênio e em condições de pH neutro ou alcalino (Guerinot & Yi, 1994). As plantas requerem aproximadamente 10^{-8} M de ferro na solução do solo para que o mesmo possa ser absorvido, mas em solos calcáreos – com pH alcalino, a quantidade de ferro solúvel não atinge 10^{-17} M (Guerinot & Yi, 1994).

A deficiência de ferro afeta diversos aspectos do desenvolvimento das plantas, como a geração de zonas cloróticas intervenais e a supressão do crescimento do meristema apical (Larcher, 2003), estando também associada à senescência em folhas (Sperotto *et al.,* 2007) e em raízes (Sperotto *et al.,* 2008).

O movimento de solutos de baixa massa molecular (como íons, ácidos orgânicos, etc.) presentes no solo até as paredes celulares de células individuais ou raízes, é um processo passivo, sem gasto energético, conduzido por difusão, ou através de transporte em massa (Marschner, 1995). A barreira constituída pela endoderme trata-se então da primeira barreira seletiva para a absorção ativa de nutrientes, como por exemplo, o ferro.

Em condições de suficiência, plantas reduzem Fe^{3+}-quelatos e transportam o Fe^{2+} resultante através da membrana plasmática via um transportador de baixa afinidade, ainda não caracterizado em nível molecular (Curie & Briat, 2003). Em condições de deficiência de ferro as plantas desenvolveram diferentes estratégias para aumentar a absorção deste nutriente:

- **Estratégia I**: empregada por todas as plantas superiores, exceto gramíneas. Estas plantas acidificam o solo através da ativação de uma H^+-ATPase localizada na epiderme da raiz, potencialmente codificada pelo gene *Aha2* em *Arabidopsis* (Fox & Guerinot, 1998). Através da acidificação local gerada pelo gradiente de prótons formado pela H^+ATPase, a solubilidade do ferro aumenta. Os Fe^{3+}-quelatos são então reduzidos por uma Fe^{3+}redutase específica, antes de serem transportados através da membrana plasmática da raiz por transportadores de íon ferroso (Marschner & Rohmëld, 1994). Foi identificado em *Arabidopsis* o gene *FRO2,* que codifica a Fe^{3+}redutase, regulado positivamente em condições de deficiência de ferro (Robinson *et al.*, 1999), assim como o transportador de íon ferroso, codificado pelo gene *IRT1*, igualmente regulado positivamente em condições de deficiência de ferro (Eide *et al.*, 1996).

- **Estratégia II**: estratégia utilizada pelas gramíneas. Estas plantas fazem uso da liberação de fitossideróforos na rizosfera. Os fitossideróforos são pequenas moléculas que possuem alta afinidade por ferro (Mori, 1999), como o ácido mugineico. Após a ligação entre os fitossideróforos e os átomos de ferro, ocorre o seu transporte através de um transportador específico localizado na membrana plasmática da epiderme da raiz, codificado pelo gene *YS1* em milho (Curie *et al.*, 2001). A secreção de fitossideróforos na rizosfera é fortemente induzida em condições de deficiência de ferro, bem como a indução de enzimas envolvidas na sua biosíntese (Mori, 1999). Em cevada foi identificado um

transportador ABC (*HvIDI7*), localizado no tonoplasto das células de raízes, cuja abundância encontra-se fortemente relacionada com o estado nutricional da planta, sendo especificamente induzido em condições de deficiência de ferro e não de outros metais (Yamaguchi *et al.*, 2002). Ainda não foram descritos transportadores ABC envolvidos no transporte de íons na forma inorgânica; assim o papel de *HvIDI7* pode estar relacionado ao transporte de outras moléculas como o citrato e o malato (compostos exudados para a rizosfera, que atuam semelhantemente aos fitossideróforos) em condições de deficiência de ferro.

O transporte do ferro depois de absorvido do solo é realizado via xilema através da via transpiratória. Ácidos orgânicos, como o ácido cítrico, são as principais moléculas quelantes de metal encontradas no xilema (Cataldo *et al.*, 1988). Em arroz, o transporte via xilema do complexo Fe-citrato é realizado através da proteína *FRDL1*, recentemente identificada (Yokosho *et al.,* 2008). Quando localizado nas folhas, o complexo Fe-citrato é reduzido para o posterior transporte por proteínas transportadoras de cátions divalentes através da membrana plasmática.

O ferro possui baixa mobilidade no floema, entretanto o ácido nicotinamínico (NA) é tido como um transportador de íon ferroso do floema, assim como no interior da célula e em tecidos reprodutivos (Takahashi *et al.*, 2003). O NA é um ácido orgânico que possui alta afinidade por Fe^{2+}, encontrado em todas as plantas superiores.

Aliado ao transporte realizado através da complexação com NA, o ferro é descrito como sendo transportado através do floema por polipeptídeos e proteínas. Foi identificada em *Ricinus communis* uma proteína de 2,4 kDa que se liga especificamente a Fe^{3+} e não a Fe^{2+} , denominada ITP (*Iron transport protein*), capaz de ligar-se igualmente a outros metais como Cu^{2+}, Zn^{2+} e Mn^{2+} (Krüeger *et al.*, 2002).

A subseqüente compartimentalização do ferro em organelas, como os vacúolos, plastídeos e mitocôndrias, pode então ser realizada por transportadores de metais divalentes, e sua alocação é direcionada para organelas responsáveis pelo seu uso imediato ou pelo seu armazenamento.

Em condições de excesso de ferro, os vacúolos são tidos como os responsáveis pelo seu aprisionamento (Curie & Briat, 2003). Em mutantes de ervilha que superacumulam ferro, a concentração de NA aumenta e encontra-se principalmente no interior dos vacúolos, enquanto que em condições normais e de deficiência de ferro, o NA é encontrado somente no citoplasma da célula (Pich *et al.*, 2001), indicando um importante papel de mecanismos de transporte de NA ligado a ferro na resistência a níveis tóxicos de ferro em plantas (Curie *et al.*, 2001). Os representantes da família gênica Yellow Stripe (YS) são importantes candidatos a transportadores de Fe-NA (Schaaf *et al.*, 2004). A remobilização do ferro estocado no interior dos vacúolos para as necessidades celulares foi descrita em levedura, sendo parcialmente mediada pelo gene *Smf3p* (Curie & Briat, 2003). Foi identificado em *Arabidopsis* um transportador de íon ferroso localizado no tonoplasto, chamado de *AtNRAMP3*, que possivelmente está relacionado com a remobilização do ferro localizado no vacúolo para o metabolismo celular (Thomine *et al.*, 2003).

A maior parte do ferro encontrado no interior das células vegetais é localizado no interior do cloroplasto (Terry & Low, 1982), envolvido no processo fotossintético (na biossíntese das moléculas de clorofila e na ferrodoxina). No interior dos plastídeos encontra-se localizada a ferritina, proteína globular multimérica responsável pelo sequestramento e liberação do ferro (Briat *et al.*, 1999), atuando como um tampão celular de ferro. A ferritina possui 24 subunidades formando uma esfera oca, em cujo interior podem ser estocados até 4500 átomos de ferro (Briat & Lobréaux, 1997). O ferro

complexado à ferritina representa mais de 90% de todo ferro encontrado no embrião da semente de ervilha, atuando como fonte de reserva de ferro para o desenvolvimento do aparato fotossintético da plântula após a germinação (Marentes & Grusak, 1998). No genoma de *Arabidopsis thaliana* foram identificados quatro genes correspondentes a ferritina, sendo que a expressão de duas cópias (*AtFer1* e *AtFer3*) é induzida em condições de excesso de ferro (Petit *et al.*, 2001a). Na região promotora do gene *AtFer1* foi identificada uma região IDRS (*Iron Dependent Responsive Sequence*), responsável pela indução da transcrição quando exposta a condições de excesso de ferro e pela repressão da transcrição de *AtFer1* em níveis normais e de deficiência de ferro (Petit *et al.*, 2001b). Interessantemente, o gene *AtFer1* apresenta igualmente indução de sua expressão quando a planta é exposta a H_2O_2 (Petit *et al.*, 2001a), evidenciando uma possível função da ferritina em mecanismos celulares de defesa a estresse oxidativo. De fato, a expressão ectópica de ferritina em *Nicotiana tabacum* foi responsável por tornar a planta tolerante a extresse oxidativo e a infecção por patógenos (Deák *et al.*, 1999), provavelmente por capturar o ferro livre dentro da célula e impossibilitando a nova formação de radicais livres via reação de Fenton. A importância da proteína ferritina na proteção ao estresse oxidativo mediado por ferro foi recentemente demonstrada por Ravet *et al*. (2008), que mostrou que as ferritinas são essenciais para a proteção das células e que a sua falta leva a uma redução no crescimento e defeitos no desenvolvimento reprodutivo, provavelmente devido à toxidez por excesso de ferro.

Recentemente, com o anúncio do fim do seqüenciamento do genoma do arroz, tanto da variedade *indica* (Yu *et al.*, 2002) como da variedade *japonica* (Goff *et al.*, 2002), foi possível realizar, pelo nosso grupo, buscas de genes relacionados com a homeostase de ferro no genoma de arroz. Foram identificados dezoito genes relacionados à família gênica

Yellow Stripe, dois relacionados com a família gênica FRO, onze genes relacionados com a família gênica ZIP/IRT, oito genes relacionados com a família gênica NRAMP e dois genes relacionados com a família gênica das ferritinas (Gross *et al.*, 2003). A identificação de genes envolvidos nas duas estratégias de absorção de ferro, utilizadas pelas dicotiledôneas e pelas gramíneas, no genoma do arroz, evidencia uma possível inter-relação dos genes envolvidos tanto na estratégia I como na estratégia II. De fato, recentemente foi demonstrado que plantas de arroz são capazes de absorver tanto Fe^{3+} ligado a fitossideróforos (utilizando a estratégia II), bem como Fe^{2+} (via estratégia I), não envolvendo uma Fe^{3+}redutase (Ishimaru *et al.*, 2006).

Igualmente, o papel desempenhado pelos membros das famílias gênicas pode ser diverso dentro das plantas, atuando em diferentes membranas ou na absorção de diferentes metais, tendo em vista que a especificidade de cada transportador é muito variável, como no caso do transportador *IRT1* de *Arabidopsis* (Rogers *et al.*, 2000).

Objetivos

Objetivo Geral

- Caracterizar os efeitos do excesso de ferro em distintos genótipos de arroz, identificando potenciais mecanismos de tolerância.

Objetivos específicos

- Analisar as respostas fisiológicas (atividade fotossintética, crescimento e marcadores bioquímicos de estresse) ao excesso de ferro em plantas de distintos genótipos cultivadas a campo e em laboratório;
- Determinar o papel das enzimas antioxidantes catalase, superóxido dismutase e ascorbato peroxidase na resposta ao excesso de ferro em arroz;
- Avaliar o transcriptoma de raízes de plantas de arroz, com ênfase em genes associados às respostas ao excesso de ferro, em duas cultivares, sensível e tolerante.
- Analisar o perfil de expressão dos genes de ferritina (*OsFER1 e OsFER2*) em diferentes órgãos e frente a diferentes moléculas;
- Analisar a influência da disponibilidade de ferro (deficiência e excesso) sobre a expressão de genes relacionados à homeostase de ferro em arroz, com ênfase em transportadores de ferro.

Capítulo 1

“Distinct physiological responses of two rice cultivars submitted to iron toxicity under field conditions”

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Short Running Title**: Iron toxicity in field-cultivated rice**

Distinct physiological responses of two rice cultivars subjected to iron toxicity under field conditions

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Abstract

Iron toxicity is recognized as the most widely distributed nutritional disorder in lowland and irrigated rice, derived from the excessive amounts of ferrous ions released by the reduction of iron oxides in flooded soils. Rice cultivars with variable degrees of tolerance to iron toxicity have been developed and cultural practices such as water management and fertilization can be used to reduce its negative impact. However, due to the complex nature of iron toxicity, few physiological data concerning tolerance mechanisms to excess iron in field conditions are available. In order to analyze the physiological responses of rice to iron excess in field conditions, areas with recognized history of iron toxicity (in Camaquã-RS, Brazil) and without iron toxicity (in Cachoeirinha –RS, Brazil) were used in an experiment with two rice cultivars with distinct tolerance to iron toxicity (BR-IRGA 409 - susceptible, IRGA 420 – tolerant). Plants from the susceptible cultivar grown in the iron-toxic site showed lower levels of chlorophylls and soluble proteins (along with higher carbonyl levels) indicating photo-oxidative and oxidative damage. The toxic effects observed were due to the accumulation of high levels of iron, and not due to any indirectly induced shoot deficiency of other nutrients. Higher activities of anti-oxidative enzymes were also observed in leaves of plants from the susceptible cultivar only in the iron-toxic site, probably as a result of oxidative stress, rather than a tolerance mechanism. There was no difference between cultivars in iron accumulation in the symplastic and apoplastic space of leaves, with both cultivars accumulating 85-90% of total leaf iron in the symplast. However, BR-IRGA 409 plants accumulated higher levels of iron in low molecular mass fractions than IRGA 420 plants. The accumulation of iron in the low molecular mass fraction probably has a direct influence on iron toxicity, and the tolerance mechanism used

by IRGA 420 plants may rely on their capacity to buffer the iron amounts in the low mass fraction.

Introduction

Iron toxicity is a complex and major nutritional disorder constraint affecting rice production in irrigated and rain fed lowland soils. In acid sulphate soils, high amounts of reduced iron (Fe^{2+}) become available and soluble due to the anoxic and reductive environment created by waterlogging (Ponnamperuma, 1972). Iron toxicity symptoms vary with cultivars and are characterized by a reddish-brown, purple bronzing, yellow or orange discoloration of the lower leaves (Sahrawat, 2004). Rice yield losses associated with the appearance of iron toxicity symptoms commonly range from 15-30% of total yield, depending on the cultivar and the severity of toxicity. However, in the case of severe toxicity, complete crop failure can occur (Audebert & Sahrawat, 2000).

Despite the great progress in understanding the conditions that lead to the occurrence of iron toxicity in rice, the interaction between iron excess and different rice genotypes in the field is poorly understood (Sahrawat, 2004). Several reports identified the main effects of iron toxicity in rice, and at least two distinct types of toxicity have been described in the literature: a true (or real) iron toxicity – characterized by the accumulation of toxic levels of iron in the plant body (Sahrawat, 2000; Olaleye *et al.*, 2001) and an indirect toxicity – a multiple nutritional disorder, caused by deficiency of other nutrients like K, P, Ca and Mg. Several other factors, such as hydrogen sulfide, organic acids and other reduction products in the soil solution take part in the complex phenomenon of iron toxicity (Tadano &

Yoshida, 1978), influencing the physiological status of the plant and leading to susceptibility of rice plants.

Rice cultivars with variable degrees of tolerance to iron toxicity have been developed by breeding (Fageria & Rabelo, 1987; Sahrawat *et al.*, 1996), and cultural practices such as alternative planting date, ridge planting, water management and the use of fertilizers (Benckiser *et al.*, 1984; Winslow *et al.*, 1989) have been developed. However, due to the diversity in environmental conditions where iron toxicity is expressed, none of those options is universally applicable or efficient (Becker & Asch, 2005).

Several factors involved in varietal tolerance to iron toxicity have been identified in distinct rice cultivars and sites (Sahrawat, 2004). Among them, the involvement of the root exclusion power, oxidizing iron at the root surface and leading to the generation of the iron plaque, has been suggested as a mechanism used by tolerant rice cultivars to exclude high amounts of iron in the soil solution from the plant body (Ando *et al.*, 1983; Green & Etherrington, 1977). Besides taking part in tolerance mechanisms, the accumulation of iron in the form of the ferric hydroxides goethite and lepidocrocite in rice roots (Bacha & Hossner, 1977) has a controversial effect. The chemical and physical properties of the iron plaque are similar to iron oxides in the soil, being able to adsorb nutrients, especially phosphorus (Kuo, 1986). Therefore, higher iron precipitation in rice roots could influence the uptake of important nutrients, possibly causing nutrient deficiencies (Howeler, 1973; Armstrong & Armstrong, 1988).

Mechanisms of leaf tissue tolerance to high levels of iron, with the induction of the leaf anti-oxidative system, have been suggested as an important feature used by rice cultivars to tolerate high levels of iron in shoots (Wu *et al.*, 1998; Yamauchi & Peng, 1995).

Since the identification of these tolerance mechanisms were independently done, using distinct rice cultivars and different methodological approaches, mostly in laboratory, few physiological data are available concerning the mechanisms involved in varietal tolerance (or susceptibility) to iron toxicity and its direct relation with field conditions.

In the present study, two rice cultivars varying in tolerance to iron toxicity (BR-IRGA 409- susceptible; IRGA 420- tolerant) were cultivated in an area with recognized history of iron toxicity and in a control area. Nutrient concentration, chlorophyll, soluble protein, oxidative damage to lipids and to proteins, iron accumulation in the leaf tissue and the activity of anti-oxidative enzymes were evaluated. Possible mechanisms involved in susceptibility and tolerance to iron toxicity and their relation with chemical characteristics of the soil were discussed.

Materials and Methods

Soil analysis and nutrient content determination in plant material

The plant and soil samples were collected in two different sampling areas, in a site with recognized history of iron toxicity in Camaquã - RS, Brazil (30º 54' 07.96"S 51º 51' 26.25"W), and in a control site (without iron toxicity) in Cachoeirinha – RS, Brazil (29º 56' 51.91''S 51º 06' 46.36'' O)**.** The soil samples were collected in a quadrant of 1 m^2, at four points randomly chosen inside the quadrant. Samples were mixed in equal proportion, air-dried and 100 g of compound samples were designated for chemical analysis, according to Tedesco *et al.* (1995). For the quantification of iron in the soil solution, 500 g of air-dried soil were kept in distilled water for about 45 days at room temperature and protected from light (to allow iron reduction). Soil solutions were than filtered (using a 0.45 µm filter),

acidified using concentrated HCl (at a final concentration of 0.5 M HCl) and iron determined by atomic absorption spectrophotometry (Varian-Model Spectra 10/20, Victoria, AU).

Plants from two rice (*Oryza sativa* L. ssp *indica*) cultivars varying in tolerance to iron toxicity (BR-IRGA 409 - susceptible; IRGA 420 - tolerant) were collected, immediately frozen and stored for further analysis. Both cultivars have been developed by the Rice Breeding Group of the Instituto Rio Grandense do Arroz (IRGA) in Brazil, and were characterized as susceptible and tolerant to iron toxicity following the methodology proposed by Bacha & Ishiy (1996). For nutrient determination, shoots (20 g) were dried at 60°C and N, P, K, Ca and Mg were extracted with H_2O_2 and H_2SO_4. Micronutrients (Zn, Cu, Fe, Mn, B), Na and S were quantified after digestion with HNO_3–$HClO_4$, except for B, which was extracted with H_2SO_4 after burning the leaf material in a muffle furnace at 500 °C for 3 hours. Nutrient concentrations were determined according to Zandavalli *et al.* (2004).

Apoplastic, symplastic and low molecular mass (< 3 kDa) iron

Fully expanded leaves of plants cultivated in Camaquã were used for the determination of apoplastic iron, according to Nikolic & Römheld (2002). Leaf segments (0.15 cm^2 of area) were vacuum infiltrated with a solution containing 1.5 mM 2,2'-bipyridyl and 10 mM MES (pH 6.0) and then incubating for 20 min under reductive conditions by adding sodium dithionite at a final concentration of 5.0 mM under continuous N_2 bubbling. Apoplastic iron was removed as Fe^{II}[bipyridyl]$_3$ complex, and iron concentration was measured by determining the absorbance at 520 nm and using the extinction coefficient of 8.65 mM^{-1}. To determine symplastic iron, after determination of apoplastic iron, samples were washed in

distilled water, dried at 60°C and ashed at 500°C for 3 hours. The ashes were mineralized in concentrated HCl, and iron was quantified using an atomic absorption spectrophotometer (Varian-Model Spectra 10/20, Victoria, AU).

For the determination of iron in low molecular mass fractions (< 3 kDa), fully expanded leaves were ground in Chelex-treated 100 mM sodium phosphate (pH 7.0), and the leaf extracts centrifuged at 20.000 x g (10 min, 4°C). The supernatant was collected, placed into a Centricon-3 micro-concentrator (Amicon) and centrifuged at 6.800 x g for 3 h (4°C). The filtrates were diluted, acidified with bi-distilled HNO_3 and iron determined by atomic absorption spectrophotometry (Zeiss AAS5, Analytik Jena, AG).

Chlorophyll and soluble proteins

Fully expanded leaves were ground in liquid nitrogen and chlorophyll extracted in acetone 85%, quantified by measuring absorbance at 663 nm and 645 nm (spectrophotometer Cintra 5, GBC Scientific Equipment, Victoria, AU) and the concentrations of chlorophyll *a* and *b* calculated according to Ross (1974). Soluble proteins were extracted from fully expanded leaves in Tris 50 mM (pH 7.0) buffer, followed by centrifugation at 10.000 x g for 15 min (4°C). The supernatant was used for the determination of soluble protein using the dye-binding method, according to Bradford (1976), using BSA as standard.

Antioxidative Enzymes

For all enzymatic activity determinations, fully expanded leaves were ground in cold extraction buffer (50 mM HEPES (pH 7.4), 1% PVP, 1 mM EDTA and 1 mM PMSF), centrifuged at 12.000 x g for 15 min at 4°C, and the supernatants immediately used for enzymatic assays. Ascorbate peroxidase (APX) activity was determined according to

Klapheck *et al*. (1990), from the decrease in absorbance at 290 nm. Guaiacol peroxidase (G-POX) was determined according to Cakmak & Marschner (1992), following the oxidation of guaiacol at 470 nm. Catalase (CAT) activity was determined following the decrease of absorbance at 240 nm due to H_2O_2 consumption (Cakmak & Marschner, 1992) and superoxide dismutase (SOD) activity was measured as described by Beyer & Fridovich (1987), using 15 min of illumination and recording the absorbance at 560 nm. Enzymatic activities were assayed in triplicate for each biological replicate, at 25°C, with no lag period, and protein was quantified by the dye binding method (Bradford, 1976).

Oxidative Damage to Lipids and Proteins

Lipid peroxides were extracted in ethanol 80% from fully expanded leaves and lipid peroxidation determined by measuring the concentration of thiobarbituric acid-reacting-substances (TBARS) as described by Hodges *et al*. (1999). Oxidative damage to proteins was determined by the quantification of carbonyl groups, by derivatization with 2,4-dinitrophenyl-hydrazine. Fully expanded leaves were ground in cold extraction buffer (50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM PMSF and 1 mM benzamidine), centrifuged at 12.000 x g for 15 min at 4°C, and the supernatants immediately used for carbonyl determination according to Levine *et al*. (1990).

Statistical analyses

Data from all tables and figures represent averages from four biological replicates (n=4). Means corresponding to the two rice cultivars were compared independently for each growing site by student's t-test, according to Quinn & Keough (2002). Differences were considered significant when $P \leq 0.05$.

Results

Chemical analysis of soil and plant material

The chemical analyzes of soils indicated low pH values and low fertility (indicated by low values of C.E.C) in both sampling areas. However, soil from the iron-toxic site showed higher values of pH (P = 0.001) and exchangeable Ca (P = 0.001) as well as lower levels of organic matter (O.M.) (P = 0.017) and P ($P \leq 0.000$) than samples from the control site (Cachoeirinha) (Table 1). As expected, Fe concentration in the soil solution was higher in Camaquã than in Cachoeirinha ($P \leq 0.0001$).

Shoots of BR-IRGA 409 plants cultivated in Camaquã (Table 2) showed higher levels of the macronutrients N (P = 0.002), P (P = 0.016), K (P = *0.044),* Mg ($P \leq 0.001$) and S (P = 0.007), and of the micronutrients Zn (P = 0.013), and Mn ($P \leq 0.001$) in comparison to IRGA 420 plants (Table 2). The concentration of Fe in shoots of both cultivars was not statistically different (P = 0.680) in plants cultivated in Camaquã. The same pattern was not observed in plants from Cachoeirinha (Table 2), with plants from the susceptible cultivar showing lower levels of N ($P \leq 0.001$) and higher levels of P ($P \leq 0.000$), Ca (P = 0.052), Mg (P = 0.000) and Fe (P = 0.001) in comparison to IRGA 420 plants.

Iron accumulation and distribution in fully expanded leaves

No statistical difference on the distribution of iron between symplasm and apoplasm in fully expanded leaves from both cultivars could be observed (Figure 1A), with plants accumulating 85-90 % of the total detected iron in the symplast. However, plants from the susceptible cultivar accumulated higher levels of iron in the low molecular weight fractions of leaf extracts (P = 0.027) (Figure 1B).

Physiological status of rice plants

Plants from the susceptible cultivar (BR-IRGA 409) showed typical symptoms of iron toxicity (such as discoloration of leaves, and necrosis in older leaves) only when grown in the iron-toxic site (Camaquã). No visible symptom could be observed in IRGA 420 plants in Camaquã and in both cultivars in the control site (Cachoeirinha). Leaves from BR-IRGA 409 showed lower levels of chlorophyll *a* (P = 0.005), *b* (P = 0.043) and total chlorophyll (*a* + *b*) (P = 0.014) in comparison to IRGA 420 plants grown in Camaquã and also in the levels of chlorophyll *a* (P = 0.017), *b* (P = 0.016) and total chlorophyll (*a* + *b*) (P = 0.017) plants cultivated in Cachoeirinha (Table 3). Lower chlorophyll concentrations in BR-IRGA 409 plants than in IRGA 420 under control conditions has been routinely observed in our laboratory. In this experiment, the difference accounts for 27% of the total chlorophyll concentration in IRGA 420. Under iron excess, however, the chlorophyll levels in BR-IRGA 409 are 51% lower than in IRGA 420. Therefore, the impact of iron toxicity on chlorophyll levels was clearly stronger in BR-IRGA 409 plants. Plants from the cultivar BR-IRGA 409 grown in Camaquã also showed lower levels of soluble protein in fully expanded leaves (P = 0.005) (Table 3), with 81% less soluble protein in leaves than IRGA 420 plants.

Oxidative damage of proteins and lipids

Plants from the susceptible cultivar grown in the iron-toxic site (Camaquã) showed higher levels of carbonyl content in comparison to IRGA 420 plants (P = 0.004) cultivated in the same site (Figure 2A). No difference between samples from different cultivars could be observed in the TBARS content in fully expanded leaves from either area (Figure 2B).

Antioxidative enzymes activity

Fully expanded leaves of BR-IRGA 409 plants showed higher activity of the anti-oxidative enzymes CAT (P = 0.001), SOD (P = 0.009) and G-POX (P = 0.003) (Figure 3), and no difference in the activity of the enzymes could be observed between plants cultivated in Cachoeirinha.

Discussion

High concentration of iron (above 280 mg L^{-1}) in the soil solution was detected only in soil samples from Camaquã, confirming the toxicity character of the experimental site, in comparison to 29 mg L^{-1} found in soil samples from the control site, Cachoeirinha. A soil solution concentration of 300 mg L^{-1} is generally considered the critical limit for the cultivation of lowland rice (Becker & Asch, 2005). This result clearly validates the use of Cachoeirinha as a control site.

The analysis of nutrient concentrations in shoots from both cultivars indicated a direct effect of the accumulation of high levels of iron in its toxicity, and not due to any indirectly induced shoot deficiency of other nutrients. All nutrients analyzed were found in normal levels described for rice plants (Westfall *et al*., 1973), with the exception of iron, reaching critical levels of toxicity in plants cultivated in Camaquã. Silveira *et al*. (2007), using plants grown in hydroponics system and the same susceptible cultivar used in our study (BR-IRGA 409) found the same pattern, with plants accumulating higher levels of iron and not showing deficiencies of other nutrients. Interestingly, shoots from the tolerant cultivar accumulated higher levels of iron than plants from the susceptible cultivar (BR-IRGA 409) in the iron-toxic site. When cultivated under iron excess, plants from both cultivars reached

iron concentrations well above 300 μg g^{-1} DW, which is considered a limit beyond which iron toxicity symptoms can occur (Fageria *et al*., 2003). We further investigated the iron distribution within leaves from both cultivars grown under this condition.

As shown by the apoplastic and symplastic iron determinations, leaves from both cultivars accumulated 85-90 % of total iron inside the cells. This result indicates that both cultivars deal with high amounts of intracellular iron, although only plants from the susceptible cultivar developed typical symptoms of iron toxicity and suffer from oxidative stress. Also, plants from the susceptible cultivar accumulated higher amounts of iron in the low molecular mass fractions (< 3kDA) of leaf extracts. Since iron capable of ROS generation (specially the hydroxyl radical) is known to be bound to several small chelators (such as carboxylic acids, di- and tri-phosphate nucleotides - Floyd, 1983; Baker & Gebicki, 1986), the higher amounts of iron found in the low molecular mass fractions seems to be responsible for the photo-oxidative damage and the toxic effects of iron in BR-IRGA 409 plants. Since plants from the resistant cultivar accumulated lower levels of iron in the low molecular mass fractions, one possible mechanism for tolerance to iron toxicity used by this cultivar could be the storage of iron in higher mass compounds (> 3kDA). Ferritin, a globular multimeric protein able to accumulate up to 4500 atoms of iron, has been considered to play a major role in buffering the intracellular iron (Briat *et al*., 1999), and, in *Arabidopsis,* one isoform (*AtFer1*) has its expression induced by excess iron (Petit *et al*., 2001). However, no difference in ferritin accumulation in fully expanded leaves could be observed between both cultivars in Western blots (data not shown), excluding a possible relation between ferritin accumulation in leaves and the tolerance mechanism of IRGA 420 plants in the conditions tested. Besides accumulation in ferritin molecules, the incorporation of iron in essential proteins (or at least in higher mass compounds), or in

iron-containing particles precipitated in chloroplasts or in vacuoles (Becker *et al*., 1994) could be part of the mechanism used by IRGA 420 to tolerate higher levels of iron.

Concentrations of chlorophyll *a* and *b* where much lower in plants from the susceptible cultivar than in IRGA 420 plants when both where cultivated in the iron-toxic soil. In this condition, total chlorophyll concentration in BR-IRGA 409 plants was equivalent to less than half the chlorophyll concentration in the tolerant cultivar. This observation indicates that iron toxicity could be related to photo-oxidative damage in the susceptible cultivar, probably as a direct result of oxidative stress. Excessive amounts of iron can be especially toxic to photosynthetic metabolism, leading to photoinhibition, increased reduction of PSII and higher thylakoid energization in *Nicotiana plumbaginifolia* cuttings exposed to excess iron (Kampfenkel *et al*., 1995). Suh *et al.* (2002) showed that iron excess led to photodamage of PSII, derived from excessive production of singlet oxygen in pea plants. BR-IRGA 409 plants also showed lower levels of soluble proteins in fully expanded leaves in comparison to IRGA 420 plants cultivated in Camaquã. Along with the lower levels of soluble proteins, fully expanded leaves of BR-IRGA 409 plants showed higher amounts of carbonyl, an oxidative stress marker, related with the oxidation of proteins driven by oxidative stress (Stadtman, 1992). The oxidative modification of proteins driven by ROS is known to induce the rapid degradation of these modified proteins (Xiong *et al*., 2007). The lower levels of soluble proteins observed in BR-IRGA 409 leaves could be indicative of oxidative damage and thereby of degradation of oxidative-modified proteins. No difference in lipid peroxides (as indicated by TBARS amounts in fully expanded leaves) could be observed between both cultivars and areas, but, as pointed out by Becana *et al*. (1998), the oxidative modification of proteins is a more sensitive marker of oxidative stress in plants than is lipid peroxidation.

The involvement of leaf tissue tolerance (including the anti-oxidative pathways) to high levels of iron was suggested as a possible mechanism used by rice plants to tolerate iron excess (Wu *et al*., 1998). However, the activity evaluation of four anti-oxidative enzymes indicated no clear relation between the anti-oxidative metabolism and tolerance to iron excess in IRGA 420 plants. Since only BR-IRGA 409 plants from the iron toxic area showed higher activities of CAT, SOD and G-POX, the observed induction was probably a consequence of the oxidative stress generated by the higher amounts of iron in the low molecular mass fractions, rather than a mechanism used by IRGA 420 plants to achieve tolerance to iron excess.

Iron toxicity had a dramatic photo-oxidative effect on the susceptible cultivar used in this study (BR-IRGA 409), leading to low levels of soluble protein and chlorophyll and higher carbonyl content. No evident mechanism used to tolerate iron toxicity could be observed in IRGA 420 plants. Proposed mechanisms in the literature, such as the exclusion of iron in the roots (or its retention in the root system) and the leaf tissue tolerance (based on anti-oxidative enzymes) do not appear to play part in the tolerance mechanism used by this cultivar, but the capacity to avoid accumulation of iron in low mass molecular fractions appears to have a great influence in its tolerance. Therefore, the distribution of iron into distinct cellular pools and the plant's ability to regulate the iron amounts in the low mass fraction appear as new important parameters to be considered in relation to tolerance to iron toxicity. Continuing efforts to characterize the physiological responses of rice plants from field experiments will probably bring into light other elements important in iron toxicity and tolerance in different rice cultivars.

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Table 1. Chemical analysis of soils from the two distinct sites used. Each value represents the mean of four replicate analyses ± standard deviation (n=4). O.M.= organic matter; C.E.C.= cation exchangeable content; exch = exchangeable. (**P* ≤ 0.05 by student's *t*-test)

	Camaquã	Cachoeirinha
pH (H_2O)	5.23 ± 0.13 *	4.8 ± 0.06
P (mg dm^{-3})	3.88 ± 0.13	14 ± 0.7 *
K (mg dm^{-3})	69.00 ± 1.3	48 ± 0.9
O.M. (%)	0.53 ± 0.05	0.65 ± 0.03 *
Ca_{exch} (cmol dm^{-3})	2.9 ± 0.14 *	2.3 ± 0.06
Mg_{exch} (cmol dm^{-3})	1.68 ± 0.1	1.5 ± 0.6
C.E.C. (cmol dm^{-3})	9.33 ± 0.66	8 ± 0.12
Fe (mg L^{-1})	284.36 ± 1.17 *	29.4 ± 1.2

Table 2. Nutrient concentration from shoots of rice plants cultivated in an iron-toxic (Camaquã-RS) and in a control site (Cachoeirinha-RS) in Brazil. Values represent the means of four replicate analysis (n=4). (* $P \leq 0.05$ by student's *t*-test)

	Camaquã		Cachoeirinha	
	IRGA 420	BR-IRGA 409	IRGA 420	BR-IRGA 409
N (%)	1.2 ± 0.07	1.7 ± 0.07 *	2.97 ± 0.02 *	2.57 ± 0.03
P (%)	0.25 ± 0.07	0.28 ± 0.05 *	0.34 ± 0.03 *	0.31 ± 0.03
K (%)	1.2 ± 0.4	2.25 ± 0.09 *	1.8 ± 0.04	1.87 ± 0.03
Ca (%)	0.16 ± 0.05	0.16 ± 0.06	0.28 ± 0.01	0.30 ± 0.01 *
Mg (%)	0.15 ± 0.05	0.21 ± 0.06 *	0.16 ± 0.05	0.20 ± 0.07 *
S (%)	0.10 ± 0.05	0.14 ± 0.02 *	0.21 ± 0.03	0.2 ± 0.03
Cu (µg g^{-1} DW)	3 ± 0.9	5.75 ± 2.1	19.5 ± 7.88	20.5 ± 11.55
Zn (µg g^{-1} DW)	21 ± 0.41	26 ± 1.08 *	21.25 ± 0.63	20.75 ± 0.75
Fe (µg g^{-1} DW)	404.75 ± 19.46	385.5 ± 39.02	186.75 ± 1.97	235.25 ± 5.38 *
Mn (µg g^{-1} DW)	492.5 ± 10.87	712 ± 19.27 *	186.75 ± 2.87	183.25 ± 1.25
B (µg g^{-1} DW)	3.5 ± 0.28	4 ± 0.25	5.5 ± 0.29	5.75 ± 0.25

Table 3. Physiological status of rice plants cultivated in an iron-toxic (Camaquã-RS) and in a control site (Cachoeirinha-RS) in Brazil. Values represent the means of four replicate analysis (n=4). (*$P \leq 0.05$ by student's *t*-test)

	Camaquã		Cachoeirinha	
	IRGA 420	BR-IRGA 409	IRGA 420	BR-IRGA 409
Chlorophyll *a* (mg g^{-1}DW)	6.46±0.44 *	3.43±0.12	8.37±0.50 *	6.24±0.18
Chlorophyll *b* (mg g^{-1}DW)	2.92±0.54 *	1.10±0.40	2.94±0.20 *	2.01±0.05
Total chlorophyll (mg g^{-1}DW)	9.36±0.95 *	4.53±0.16	11.31±0.70 *	8.24±0.23
Soluble protein (mg g^{-1}DW)	255.85±33.90 *	31.17±8.80	217.35 ±8.82	225.64±8.40

Figure Legends

Figure 1. Iron accumulation and distribution in fully expanded leaves of rice plants cultivated in Camaquã-RS, Brazil. (A) Symplastic (dark bars) and apoplastic (gray bars) iron in leaves of rice plants and (B) Iron accumulation in low molecular weight fractions (< 3kDa) in fully expanded leaves of rice plants. Each value represents the mean of four replicates ± standard deviation (n=4). (*$P \leq 0.05$ by student's t-test)

Figure 2. Oxidative damage to proteins (A) (indicated by carbonyl content) and lipids (B) (indicated by TBARS. 3B) of fully expanded leaves of rice plants from cultivars IRGA 420 (dark bars) and BR-IRGA 409 (gray bars) from two distinct sampling sites (Cachoeirinha and Camaquã). Each value represents the mean of four replicates ± standard deviation (n=4). (*$P \leq 0.05$ by student's t-test)

Figure 3. Activity of the antioxidant enzymes (A) CAT. (B) G-POX. (C) SOD and (D) APX from fully expanded leaves of rice plants from cultivars IRGA 420 (dark bars) and BR-IRGA 409 (gray bars) from two distinct sampling sites (Cachoeirinha and Camaquã). Each value represents the mean of four replicates ± standard deviation (n=4). (*$P \leq 0.05$ by student's t-test)

Figure 1

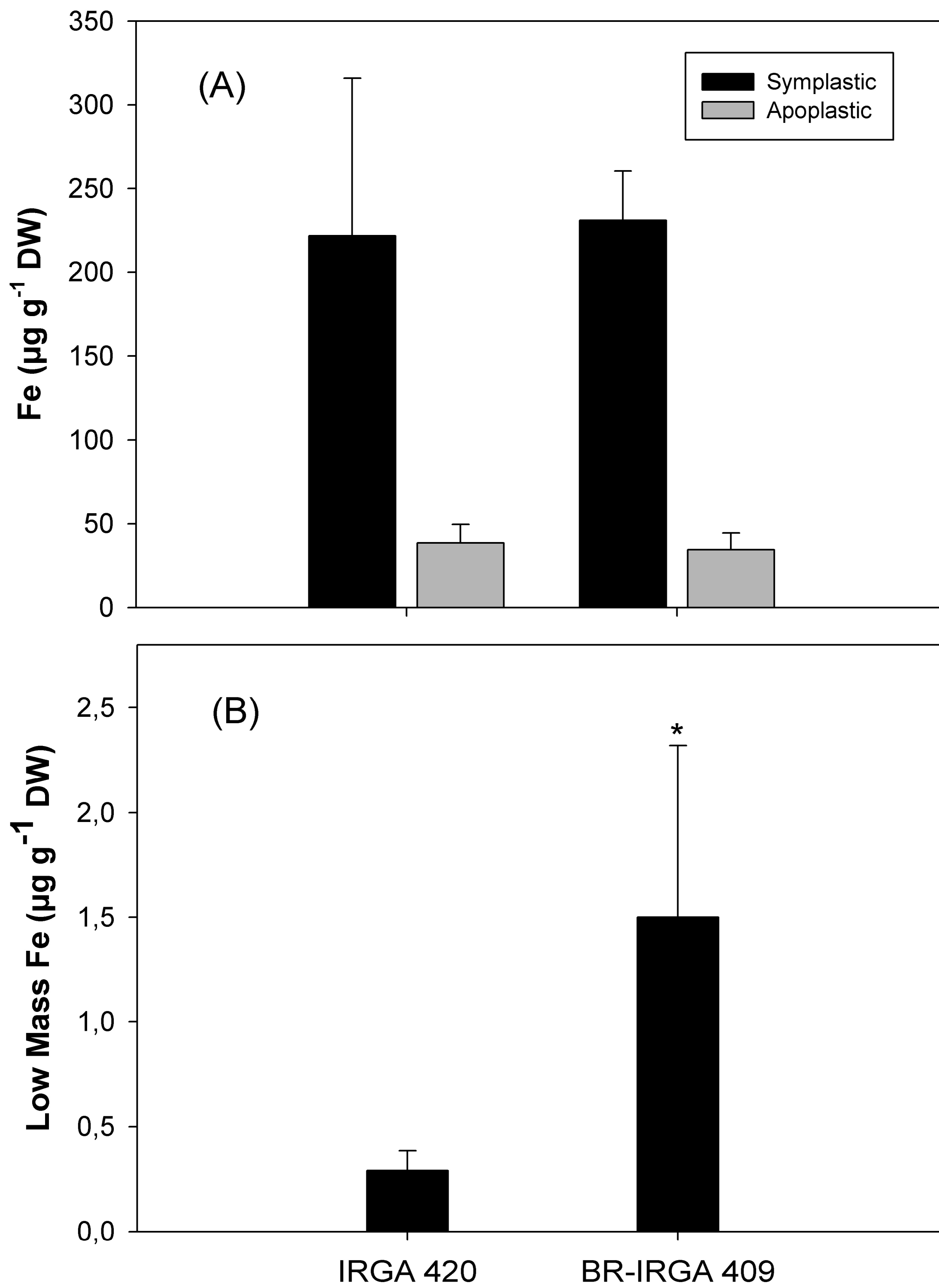


Figure 2

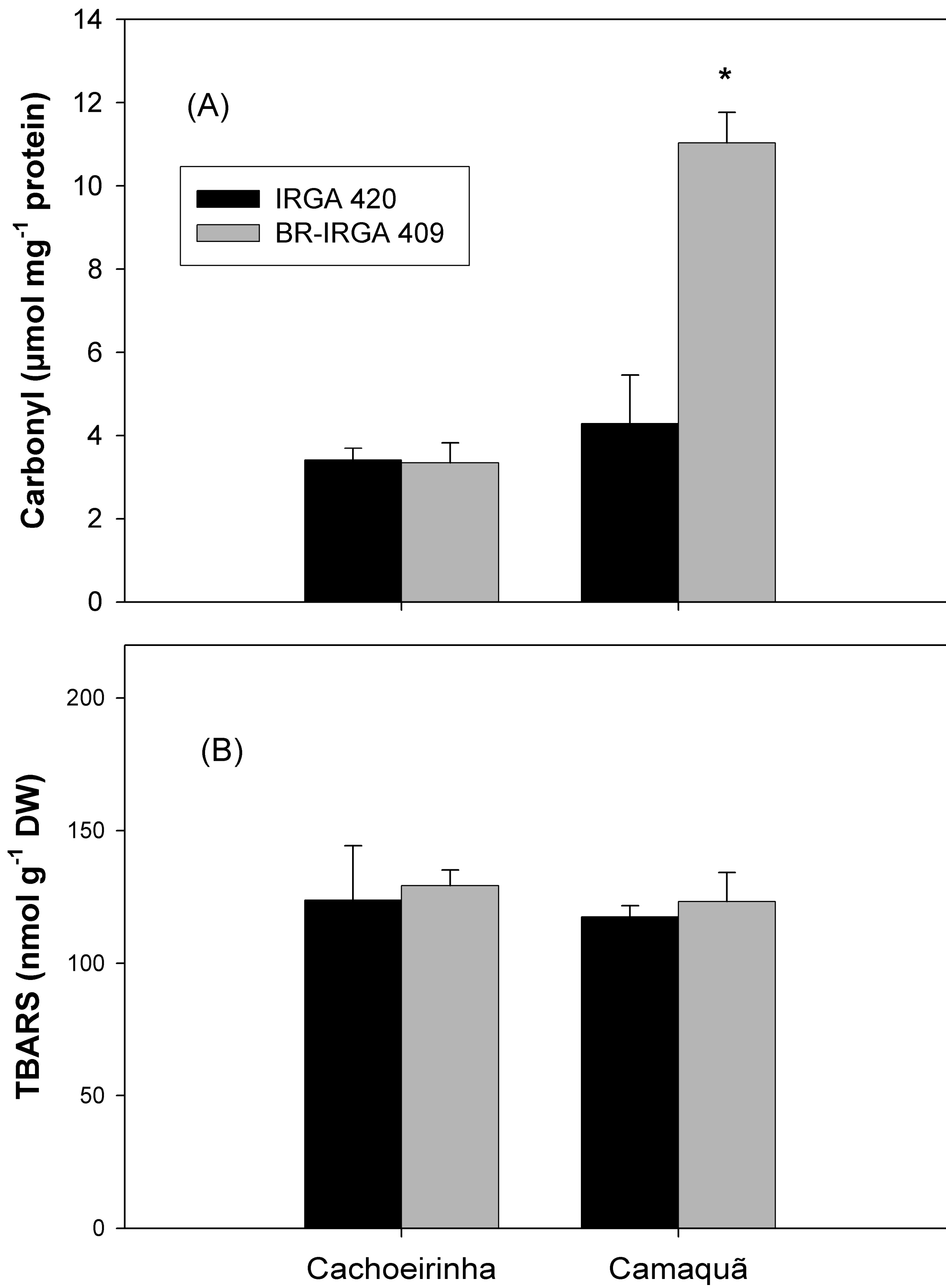
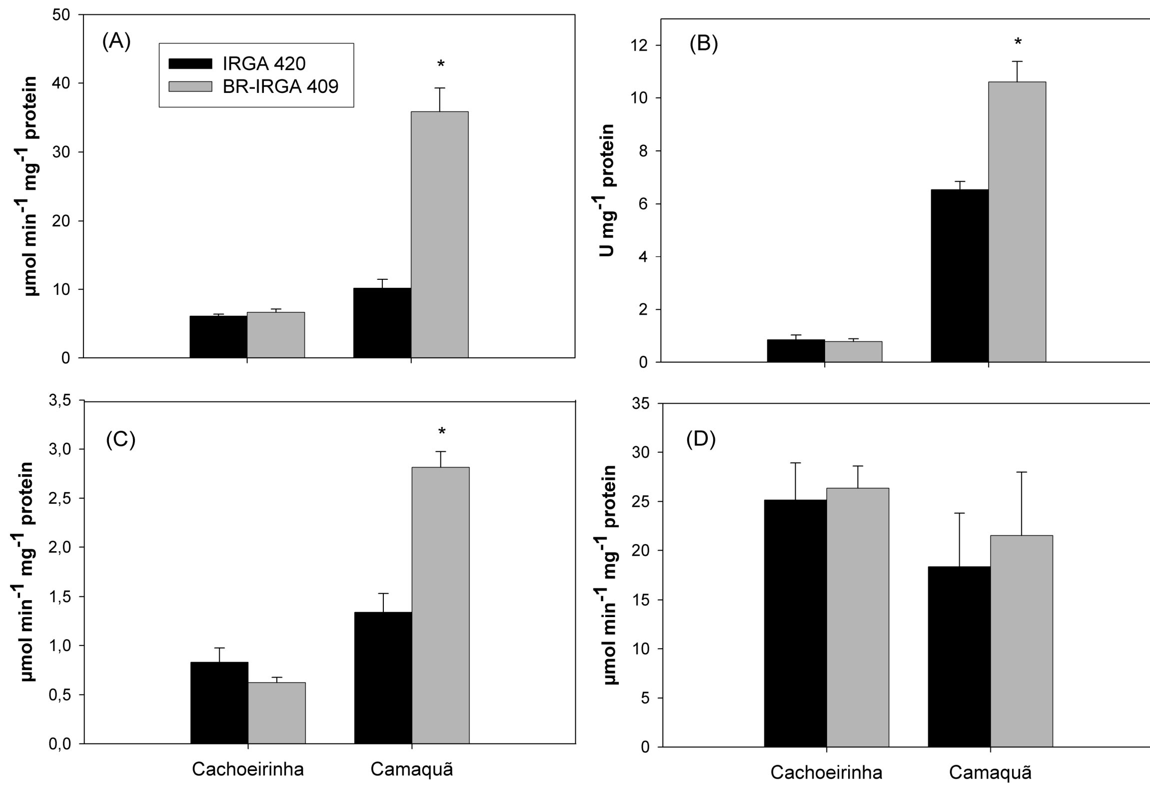


Figure 3



Capítulo 2

“Iron toxicity in rice: diverse tolerance mechanisms in distinct cultivars”

Short Running Title: **Tolerance to iron toxicity in rice.**

Iron toxicity in rice: diverse tolerance mechanisms in distinct cultivars

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Abstract

Iron toxicity is a common problem in waterlogged and lowland rice. Tolerance to iron toxicity has been a subject of several studies, but few data about the mechanisms used by distinct rice cultivars in the field are available. To analyze the responses of different cultivars to iron toxicity, we studied the physiological responses of three distinct cultivars BR-IRGA 409 (susceptible to iron toxicity), EPAGRI 108 and EPAGRI 109 (two tolerant cultivars) grown at two distinct sites – an iron-toxic (Camaquã-RS, Brazil) and a control site (Cachoeirinha-RS, Brazil). Plants from the susceptible cultivar (BR-IRGA 409) showed visible symptoms of iron toxicity only when grown at the iron-toxic site (Camaquã) with lower levels of chlorophyll and higher oxidative damage to proteins. As expected, plants from the iron-toxic site accumulated higher levels of iron, and iron accumulation and concentration greatly differed among the studied cultivars. EPAGRI 109 plants accumulated iron at levels similar to BR-IRGA 409 in leaves, while EPAGRI 108 showed lower levels of iron, suggesting that the two tolerant cultivars displayed distinct tolerance mechanisms to iron toxicity. To further detail the iron accumulation among the cultivars, we analyzed the accumulation in DCB (dithionite-citrate-bicarbonate)-treated roots and non treated roots. Interestingly, the cultivar with the lower iron concentration in leaves accumulated the higher levels of iron in its DCB-treated roots, suggesting that the capacity to accumulate iron in roots could be a part of the mechanism used by this cultivar. The leaf antioxidant capacity (based on the activity of the enzymes SOD, APX, CAT, GR and DHAR and the ascorbate and glutathione accumulation) also greatly varied between the genotypes. Plants from the susceptible cultivar grown in the iron-toxic site showed higher activity of APX and higher DHA and GSSG concentration (the oxidized forms of ascorbate and glutathione, respectively), confirming that this cultivar suffers from oxidative stress,

while plants from the tolerant cultivar that accumulated similar iron levels (EPAGRI 109) showed the higher SOD, GR and DHAR activities. The remarkably higher SOD activity seen in this cultivar, along with increased GR and DHAR activities, could be directly involved with its capacity to tolerate high levels of iron in the leaf tissue. We further investigated the SOD gene expression by evaluating the specific expression of four SOD isoforms (one MnSOD, two Cu/ZnSODs and one FeSOD). Only EPAGRI 109 plants showed higher mRNA abundance of three from the four SOD genes tested, evidencing differential regulation among the tested SOD genes. Our results suggest that tolerance to iron toxicity can vary among distinct rice cultivars even when grown at the same site, and that the accumulation of iron in the roots, limiting its translocation to the shoot, as well as the leaf tissue tolerance are both important features in the capacity to tolerate iron toxicity in field conditions.

Introduction

Iron toxicity is a major nutritional disorder in lowland and waterlogged rice. While it may occur in a wide range of soil types, general characteristics of most of the iron-toxic soils are high amounts of reducible iron, low pH, and low cation exchange capacity and exchangeable K content (Ottow *et al.*, 1982). Most importantly, iron toxicity is linked to water logging and only occurs under anoxic soil conditions, with the reduction of iron oxides and its solubilization on the soil solution (Ponnamperuma, 1972). Losses in rice productivity may commonly vary between 15-20%; however, in most severely cases, complete crop failure can occur (Audebert & Sahrawat, 2000; Winslow *et al.*, 1989).

Rice cultivars with variable degrees of tolerance to iron toxicity have been obtained by breeding (Fageria & Rabelo, 1987; Sahrawat *et al*., 1996), and cultural practices such as alternative planting date, ridge planting, water management and the use of fertilizers (Benckiser *et al.*, 1984; Winslow *et al*., 1989) have been developed. However, due to the diversity in environmental conditions where iron toxicity occurs, none of those options is universally applicable or efficient (Becker & Asch 2005). Therefore, the study of the physiological impacts of iron excess in rice plants is of a great importance for the culture and for the understanding of the conditions involved in iron toxicity and tolerance.

Despite significant progress in understanding the conditions that lead to the occurrence of iron toxicity, the interaction between iron excess and different rice genotypes in the field is poorly understood (Sahrawat, 2004). Among the possible mechanisms used by distinct rice cultivars to tolerate high levels of iron, the involvement of the root exclusion power, oxidizing iron at the root surface and leading to the formation of the iron plaque, has been suggested as a mechanism used by tolerant rice cultivars to exclude high amounts of iron in the soil solution from the plant body (Ando *et al*., 1983; Green & Etherrington, 1977). Thus, avoiding higher iron accumulation in the leaf tissue. Another possible mechanism involved in the limitation of excessive uptake of iron could be the regulation of its uptake, as seen by Silveira *et al.* (2007) working with nutrient solutions in laboratory.

The mechanisms of leaf tissue tolerance to high levels of iron, with the induction of the leaf antioxidant system, have been suggested as another important feature used by rice cultivars to tolerate high levels of iron in shoots (Wu *et al*., 1998; Yamauchi & Peng, 1995) as well as its accumulation in higher mass fractions (Stein *et al*., 2008). Since these tolerance mechanisms were independently identified, using distinct rice cultivars and different methodological approaches (with a high soil heterogeneity), few physiological data

concerning the mechanisms involved in varietal tolerance (or susceptibility) to iron toxicity and the comparison of different tolerant cultivars and its relation with field conditions are available.

In this work we described the identification and the analysis of distinct tolerance mechanisms to iron excess used by related cultivars grown in an iron-toxic and a control site.

Materials and Methods

Site and plant material

Rice plants (*Oryza sativa* ssp. *indica*) from cultivars EPAGRI 108, EPAGRI 109 (both cultivars tolerant to iron excess) and BR-IRGA 409 (susceptible) were grown in two distinct sites in Brazil, in an iron-toxic site with recognized history of iron toxicity in Camaquã - RS, Brazil (30º 54' 07.96"S 51º 51' 26.25"W), and in a control site (without iron toxicity) in Cachoeirinha – RS, Brazil (29º 56' 51.91''S 51º 06' 46.36'' W). Plants were collected, separated in shoots and roots, immediately frozen and stored at -20ºC until further analyses. The rice cultivars used in this study were developed by the Rice Breeding Group of the Instituto Rio Grandense do Arroz (IRGA) in Brazil, were characterized as susceptible and tolerant to iron toxicity following the methodology proposed by Bacha & Ishiy (1986), and are intensively planted in Southern Brazil.

Chlorophyll determination

Fully expanded leaves were ground in liquid nitrogen and chlorophyll extracted in acetone 85%, quantified by measuring absorbance at 663 nm and 645 nm (spectrophotometer Cintra

5, GBC Scientific Equipment, Victoria, AU) and the concentrations of total chlorophyll (chlorophyll *a* + chlorophyll *b*) calculated according to Ross (1974).

Oxidative damage to proteins

The oxidative damage to proteins was determined through the quantification of carbonyl groups, by derivatization with 2,4-dinitrophenyl-hydrazine. Fully expanded leaves were ground in cold extraction buffer [50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM PMSF and 1 mM benzamidine], centrifuged at 12000 x g for 15 min at 4°C, and the supernatants immediately treated with streptomycin sulphate 10% to eliminate contaminant nucleic acids, and readily used for carbonyl determination according to Levine *et al*. (1990). The carbonyl concentration was normalized with the soluble protein concentration, determined by the dye-binding method (Bradford, 1976), using BSA as standard.

Iron determination in plant material

Iron concentration was determined in plant material of the three cultivars (EPAGRI 108, EPAGRI 109 and BR-IRGA 409) grown at the two distinct sites. Roots were collected, thoroughly washed in abundant distilled water to remove excess soil and particulate material and used for iron determination or immediately kept for 3 hours in cold DCB (dithionite-citrate-bicarbonate) solution (Taylor & Crowder, 1983) to remove the iron precipitated as an iron plaque. Samples (fully expanded leaves, DCB-treated or non-treated roots) were dried at 60°C and ashed at 500°C for 3 hours. The ashes were digested with concentrated HCl and iron was quantified by atomic absorption spectrophotometry (Varian-Model Spectra 10/20, Victoria, AU).

Ascorbate and Glutathione determination

Ascorbate (AA) and dehydroascorbate (DHA) were extracted from leaf tissue (0.5-0.6 g) with 1 ml of TCA 6%, centrifuged at 12000 x g (10 min at 4^{0}C) and the supernatant collected and immediately used for AA and DHA determination, according to Okamura (1980). Glutathione (GSH) and glutathione disulfide (GSSG) were extracted with 0.8 ml of $HClO_4$ 10% and determined according to Griffith (1980) by 5,5'-dithiobis-(2-nitrobenzoic acid)-GR recycling procedure. Changes in absorbance of the reaction mixture were measured at 412 nm and total GSH concentration was calculated from a standard curve with GSH. GSSG was determined after removal of GSH by 2-vinylpiridine derivatization. A specific standard curve with GSSG was used, and GSH determined by subtraction of GSSG from the total glutathione (GSH + GSSG) concentration.

Antioxidative enzymes activity

For all enzymatic activity determinations, fully expanded leaves were ground in cold extraction buffer [50 mM HEPES (pH 7.4), 1% PVP, 1 mM EDTA and 1 mM PMSF], centrifuged at 12000 x g (15 min at 4°C) and the supernatants immediately used for enzymatic assays. The activity of ascorbate peroxidase (APX) was determined according to Klapheck *et al.* (1990), from the decrease in absorbance at 290 nm. Catalase (CAT) activity was determined following the decrease of absorbance at 240 nm due to H_2O_2 consumption (Cakmak & Marschner, 1992). Superoxide dismutase (SOD) activity was measured as described by Beyer & Fridovich (1987), using 15 min of illumination and recording the absorbance at 560 nm. The activity of glutathione reductase (GR) was determined according to Sgherri *et al*. (1994), following the NADPH consumption at 340 nm, and the dehydroascorbate reductase (DHAR) activity determined according to Kato *et al*. (1997),

following the reduction of DHA at 265 nm. Conditions for all assays were chosen so that the rate of reaction was constant for the entire experimental period and proportional to the amount of enzyme added. All enzymatic activities were assayed in triplicate at 25°C, with no lag period, and protein was quantified by the dye-binding method (Bradford 1976).

RNA extraction and cDNA synthesis

Total RNA samples were extracted from fully expanded leaves from rice cultivars using Concert Plant RNA Purification Kit (Invitrogen), according to the manufacturer instructions. RNA quality was checked by measuring the ratio of Abs 260 nm/ Abs 280 (selecting only samples that range from 1.9 to 2.1) and integrity by electrophoresis with 1.2% agarose gels. RNA was quantified using the Quant-iT RNA Assay Kit and the Qubit Fluorometer (Invitrogen). Two micrograms of total RNA were treated with DNAse I (Invitrogen) to avoid genomic DNA contamination interference, and cDNA synthesized using M-MLV reverse transcriptase (Invitrogen) and oligo-dT (30).

Semi-quantitavive RT-PCR

Semi-quantitative RT-PCR was performed using standard conditions and PCR products analyzed only in the linear phase of amplification. Diluted cDNA (1:10 in water) were used, and specific primers for four different SOD isoforms were used (Kim *et al.*, 2007): MnSOD (L34038, 5’-GGAAACAACTGCTAACCAGGAC-3’, 5’-GCAATGTACACAAGGTCCAGAA-3’), FeSOD (AB014056, 5’-TGCACTTGGTGATATTCCACTC-3’, 5’-CGAATCTCAGCATCAGGTATCA-3’) and two Cu/ZnSOD (D852339, 5’-CAATGCTGAAGGTGTAGCTGAG-3’, 5’-GCGAAATCCATGTGATACAAGA-3’; L19435, 5’-GGTTTTGGTGCTCTTTTAGGTG-

3', 5'-GCCACTCAGGTAAAGACGAAAC-3'). The PCR products were resolved in 1.2% agarose gels and stained with ethidium bromide (0.5 μg mL^{-1}). The constitutive product of ubiquitin (Miki *et al.,* 2005) was used as control.

Statistical analysis

Means obtained for the cultivars and sampling areas were compared by analysis of variance (ANOVA), followed by Duncan test, according to Quinn & Keough (2002). Differences were considered significant when $P \leq 0.05$.

Results

Physiological characterization of rice cultivars

Plants from the susceptible cultivar (BR-IRGA 409) grown at the iron-toxic site (Camaquã) showed visible typical symptoms of iron toxicity, such as discoloration and necrosis in older leaves, not seen in the tolerant cultivars (EPAGRI 108 and EPAGRI 109). Both tolerant cultivars showed higher levels of chlorophyll in comparison to BR-IRGA 409 plants, used as an indicative of iron toxicity (Figure 1A). Also, the oxidative stress to proteins, shown as the higher carbonyl concentration seen in the fully expanded leaves from the susceptible cultivar (BR-IRGA 409, Figure 1B), validated the previous classification from the Instituto Rio Grandense do Arroz and allowed us to further analyze the physiological responses of the chosen cultivars. Moreover, no clear difference between plants grown in the control site could be seen, and thereby, the use of Cachoeirinha as a control site was validated.

Iron concentration in leaves and roots

The iron concentration in leaf and root tissues was clearly site-dependent, and there was large variation among samples (Figure 2). As expected, plants cultivated in the iron-toxic site accumulated higher levels of iron in leaves (Figure 2A) and roots (Figure 2B and 2C). A clear difference between cultivars could be seen in plants grown at the iron-toxic site. Plants from the two tolerant cultivars (EPAGRI 108 and EPAGRI 109) differed in leaf iron accumulation (Figure 2A), with EPAGRI 109 accumulating an average of 3.31 mg Fe g^{-1} DW in comparison 1.66 mg Fe g^{-1} DW from EPAGRI 108, while leaves from the susceptible cultivar (BR-IRGA 409) accumulated 3.05 mg Fe g^{-1} DW, similar to EPAGRI 109.

To detail the iron accumulation in the root tissue among the studied cultivars, we collected roots from the two distinct sites and quantified the concentration of iron in treated or in non DCB-treated roots (Figure 2B and 2C). Treatment with DCB solution greatly reduced the iron concentration in root samples, proving the effectiveness of the DCB solution in solubilizing the iron precipitated in the extraplasmatic space of the root. DCB-treated roots from EPAGRI 108 plants showed the higher iron concentration in roots in comparison to the two other cultivars grown in the iron-toxic site (Figure 2B). Taken together, these results indicate that the two tolerant cultivars differ in the iron accumulation pattern in the plant body, and while EPAGRI 109 and BR-IRGA 409 accumulated iron at similar levels, EPAGRI 108 accumulated lower levels of iron in leaves but higher levels in roots.

Antioxidative metabolism (ascorbate and glutathione)

Plants from the susceptible cultivar (BR-IRGA 409) accumulated higher levels of the oxidized forms of glutathione and ascorbate (GSSG and DHA, respectively) only when

grown in the iron-toxic site (Camaquã, Table 1). No differences in ascorbate and glutathione levels were seen among plants from the tolerant cultivars.

Antioxidative Enzymes Activity

The activities of antioxidant enzymes were remarkably different between plants grown in the iron-toxic site (Camaquã), while no difference could be seen in plants from the control site (Figure 3). Leaves from EPAGRI 109 plants showed the highest SOD, GR and DHAR activities, while BR-IRGA 409 plants showed the highest APX activity. Plants from EPAGRI 109 and BR-IRGA 409 showed similar levels of CAT activity, but at much higher rates than in EPAGRI 109 plants.

SOD gene expression in leaves

To further detail the high SOD activity seen in EPAGRI 109 leaves, we analyzed the expression profile of four distinct SOD genes – one MnSOD (L34038), one FeSOD (AB014056) and two Cu/ZnSODs (D85239 and L19435). The expression of the MnSOD and the two CuZnSOD tested were higher in EPAGRI 109 leaves from the iron-toxic soil than in the other two analyzed cultivars (Figure 4A), while no clear difference could be seen in plants cultivated in the control site (Figure 4B).

Discussion

Iron toxicity differently affected the three cultivars used in this study. To characterize the response to iron toxicity, we measured the chlorophyll and carbonyl concentration in leaves from plants grown in two distinct sites. Both parameters, chlorophyll and carbonyl

concentration, have been linked with the toxic effects of iron excess in plants (Gallego *et al*., 1996; Fang *et al*., 2001; Stein *et al*., 2008) and validate the use of the chosen cultivars, indicating that only the susceptible cultivar (BR-IRGA 409) suffered from photo-oxidative and oxidative damage.

Iron accumulation in leaves and roots greatly varied between the samples and sites used. As expected, plants grown in the iron-toxic site accumulated higher levels of iron, in comparison to plants from the control site. The soil iron concentrations from both sites were previously reported to be above 280 mg L^{-1} in the iron-toxic site (Camaquã) and 29 mg L^{-1} in the control site, Cachoeirinha (Stein *et al*., 2008). The difference between the two sites clearly impacted in the iron accumulation in the plant body. Interestingly, the two selected tolerant cultivars differ in their iron accumulation in leaves, suggesting that the two cultivars displayed distinct tolerance mechanisms. The iron accumulation in roots also varied between the studied cultivars, but in an opposite way. EPAGRI 108 plants (which accumulated lower iron levels in the leaf tissue) showed the highest iron accumulation in roots. The oxidation of iron at the root surface has been proposed as a potential mechanism used by plants to tolerate high levels of iron in the soil solution (Becker & Asch, 2005), as well as the root membrane selectively (Silveira *et al*., 2007). Using the DCB solution, we could precisely define the iron accumulation in roots, and found that proportionally, EPAGRI 108 accumulated more iron in the DCB-treated roots and not in the extraplasmatic spaces (forming the so-called iron plaque). Despite this high iron concentration in roots, EPAGRI 108 did not suffer from iron toxicity. This finding can also suggest that the studied rice cultivars differ in their iron translocation capacity. The translocation of iron throughout the plant body is known to be a crucial part of the complex iron homeostasis (Curie & Briat, 2003), and recently Yokosho *et al*. (2008) identified a rice citrate

transporter responsible for the translocation of iron localized at the pericycle cells in roots. Since EPAGRI 108 plants showed lower levels of iron in leaves and higher levels in DCB-treated roots, it is possible that the capacity to reduce the iron translocation from roots to shoots takes part in the tolerance mechanism used by this cultivar. The analysis of the Fe-citrate translocation capacity could help to clarify the precise mechanism of low iron translocation to the leaves, as well as the cellular localization of iron in the roots from this cultivar. Along with higher iron accumulation in roots, the capacity to tolerate high levels of iron could be dependent of the induction of antioxidant defenses in the leaf tissues (Wu *et al.*, 1998). We analyzed the activity of several antioxidant enzymes involved in the Ascorbate-Glutathione cycle in the three cultivars grown in the two distinct sites. Interestingly, EPAGRI 109, the tolerant cultivar that accumulated iron at similar levels to the susceptible cultivar (BR-IRGA 409), showed higher activities of SOD, GR and DHAR, enzymes known to be involved in this cycle. Thereby, the tolerance to high levels of iron found in this cultivar could be directly related to the capacity to scavenge iron-mediated oxygen free radicals.

Plants from the susceptible cultivar (BR-IRGA 409), grown in the iron-toxic site, also showed higher concentrations of DHA and GSSG, indicating a clear disturbance in the GSH/AA ratio. The GSH and AA redox state is maintained through GR, MDAR and DHAR, and they have a pivotal role in the defense against ROS-induced oxidative damage (Noctor & Foyer, 1998). The higher amounts of DHA found in BR-IRGA 409 leaves probably have a direct relationship with the higher APX activity observed in the same plants, since AA is used as a reducing agent by APX to catalyze the reduction of H_2O_2 to H_2O (Shigeoka *et al.*, 2002). While higher APX activity observed in BR-IRGA 409 plants could be directly related to the oxidative stress driven by higher levels of iron

accumulated in the leaf tissue, the higher CAT activity observed in the same cultivar and in EPAGRI 109 could be related to common effects of iron excess in both cultivars. CAT is an enzyme known to be mainly involved in the photorespiration process (Foyer *et al.*, 1994), being used by plants to deviate the energy received through the photosystems (Noctor *et al.*, 2002). Besides that, plants from the cultivar EPAGRI 109 are tolerant to iron toxicity (exhibiting higher levels of chlorophyll and lower levels of carbonyl), and BR-IRGA 409 are susceptible. A plausible explanation for the high CAT activity seen in both cultivars grown only in the iron-toxic site is that both cultivars deal with high levels of iron in their leaves. Apart from the higher activity of GR and DHAR, the capacity to tolerate high levels of iron in the leaf tissue (even higher levels than the susceptible cultivar - BR-IRGA 409) observed in EPAGRI 109 could rely in the remarkably higher SOD activity. SOD is known to be responsible for the dismutation of the superoxide anion (O_2^-), and constitute the first line of defense against ROS (Alscher *et al.*, 2002). SODs have been linked to diverse stressful conditions (Bowler *et al.*, 1994) and are found in different sub-cellular compartments. Besides different localization, the classification of SODs is dependent on the metal co-factor used by the enzyme (Alscher *et al.*, 2002). Since EPAGRI 109 plants showed higher SOD activities, we further investigated the SOD gene expression by evaluating the specific expression of four SOD isoforms (one MnSOD, two Cu/ZnSODs and one FeSOD). EPAGRI 109 plants showed higher mRNA abundance of three from the four SOD genes tested, suggesting a differential regulation among the tested SOD genes. The subcellular localization of the three genes were predicted using PSORT (http://psort.ims.u-tokyo.ac.jp) and TargetP (http://www.cbs.dtu.dk/services/TargetP) (data not shown) and suggest different localizations – in mitochondria (MnSOd – L34038), plastids (Cu/ZnSOD -D85239) and

cytoplasm (Cu/ZnSOD – L19435). Whether this prediction indicates that the capacity to tolerate iron excess in EPAGRI 109 rely in increased accumulation of SOD proteins in these sub-cellular compartments remains to be tested. Nonetheless, our results suggest that iron toxicity has a differential regulation of the SOD genes in the cultivars tested, and the tolerance mechanism could rely in higher SOD activity and SOD gene expression.

Becker & Asch (2005) categorized the iron-toxic environments into three distinct clusters, according to differences in soil types, soil iron content and the rice growth stage showing most symptoms and yield losses. Possibly, the variability observed and classified into these three clusters had a major influence in the distinct tolerance mechanisms used by rice cultivars to tolerate excess levels of iron in the soil solution and thereby, each mechanism may be more suitable to cope with specific adverse soil conditions. Our results showing that two related cultivars (EPAGRI 108 and EPAGRI 109) grown in the same sites, displayed distinct mechanisms to tolerate iron toxicity indicates that not only the conditions where iron toxicity is expressed are variable, but also the capacity to respond and to tolerate it, is complex. Different rice cultivars may face the highest iron toxic concentrations and other related stresses during different developmental stages, and may cope with the corresponding challenges in different ways, depending on the corresponding stage.

This complex interaction between the rice plant and its environment indicates that new approaches used to investigate the effects of iron excess should analyze its effects in distinct rice cultivars and its relation with the environment.

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Table 1. Accumulation of reduced (AA and GSH) and oxidized (DHA and GSSG) forms of ascorbate (AA and DHA) and glutathione (GSH and GSSG) in fully expanded leaves of three rice cultivars (EPAGRI 108, EPAGRI 109 and BR-IRGA 409) grown in an iron-toxic (Camaquã-RS, Brazil) and a control site (Cachoeirinha-RS, Brazil). Values represent the mean of six biological replicates ±standard error (n=6). Distinct letters indicate statistical difference by the Duncan test ($P \leq 0.05$). N.D.= bellow the detection limit.

	EPAGRI 108		EPAGRI 109		BR-IRGA 409	
	Camaquã	Cachoeirinha	Camaquã	Cachoeirinha	Camaquã	Cachoeirinha
AA (μmol mg^{-1} DW)	1.14±0.13b	1.18±0.05b	1.10±0.03b	1.73±0.06ab	0.84±0.07c	1.42±0.16ab
DHA (μmol mg^{-1} DW)	N.D.	N.D.	N.D.	N.D.	0.48±0.03	N.D.
GSH (μmol mg^{-1} DW)	11.22±0.67bc	12.29±0.48bc	10.58±0.68c	13.79±1.46b	11.4±1.10c	19.75±1.01a
GSSG (μmol mg^{-1} DW)	0.28±0.02c	0.31±0.02c	0.38±0.07c	0.26±0.04c	2.85±0.55a	0.50±0.03b

Figure Legends

Figure 1. Physiological characterization of tolerance to iron toxicity in three rice cultivars (EPAGRI 108, EPAGRI 109 and BR-IRGA 409). Chlorophyll concentration (A) and oxidative stress in proteins, indicated by the carbonyl concentration (B). Plants were grown in an iron-toxic site (Camaquã-RS, Brazil) or in a control site (Cachoeirinha-RS, Brazil). Values represent the means of six replicates ± standard error (n=6). Distinct letters indicate statistical difference by the Duncan test ($P \leq 0.05$).

Figure 2. Iron accumulation and distribution in leaves (A), DCB-treated roots (B), and untreated roots (C) of rice cultivars (EPAGRI 109, EPAGRI 108 and BR-IRGA 409) grown in an iron-toxic site (Camaquã-RS, Brazil) or in a control site (Cachoeirinha-RS, Brazil). Values represent the means ± standard error (n=6 for leaves; n=4 for roots). Distinct letters indicate statistical difference by the Duncan test ($P \leq 0.05$).

Figure 3. Activities of antioxidant enzymes (SOD, APX, CAT, GR and DHR) in fully expanded leaves from the rice cultivars EPAGRI 109, EPAGRI 108 and BR-IRGA 409 grown in an an iron-toxic site (Camaquã-RS, Brazil) or in a control site (Cachoeirinha-RS, Brazil). Values represent the means of six replicates ± standard error (n=6). Distinct letters indicate statistical difference by the Duncan test ($P \leq 0.05$).

Figure 4. Expression profile of SOD isoforms in fully expanded leaves from three rice cultivars (EPAGRI 109, EPAGRI 108 and BR-IRGA 409) grown in two distinct sites – an iron-toxic site (A) located in Camaquã-RS, Brazil; and a control site (B) in Cachoeirinha-

RS, Brazil. Total RNA was isolated from fully expanded leaves and used for cDNA synthesis and PCR amplification using specific primers for four rice SOD isoforms (one MnSOD, one FeSOD and two Cu/ZnSOD). The expression level of ubiquitin (*OsUbq*) was used as control. The experiments were repeated twice with independent RNA samples, and similar results were obtained.

Figure 1

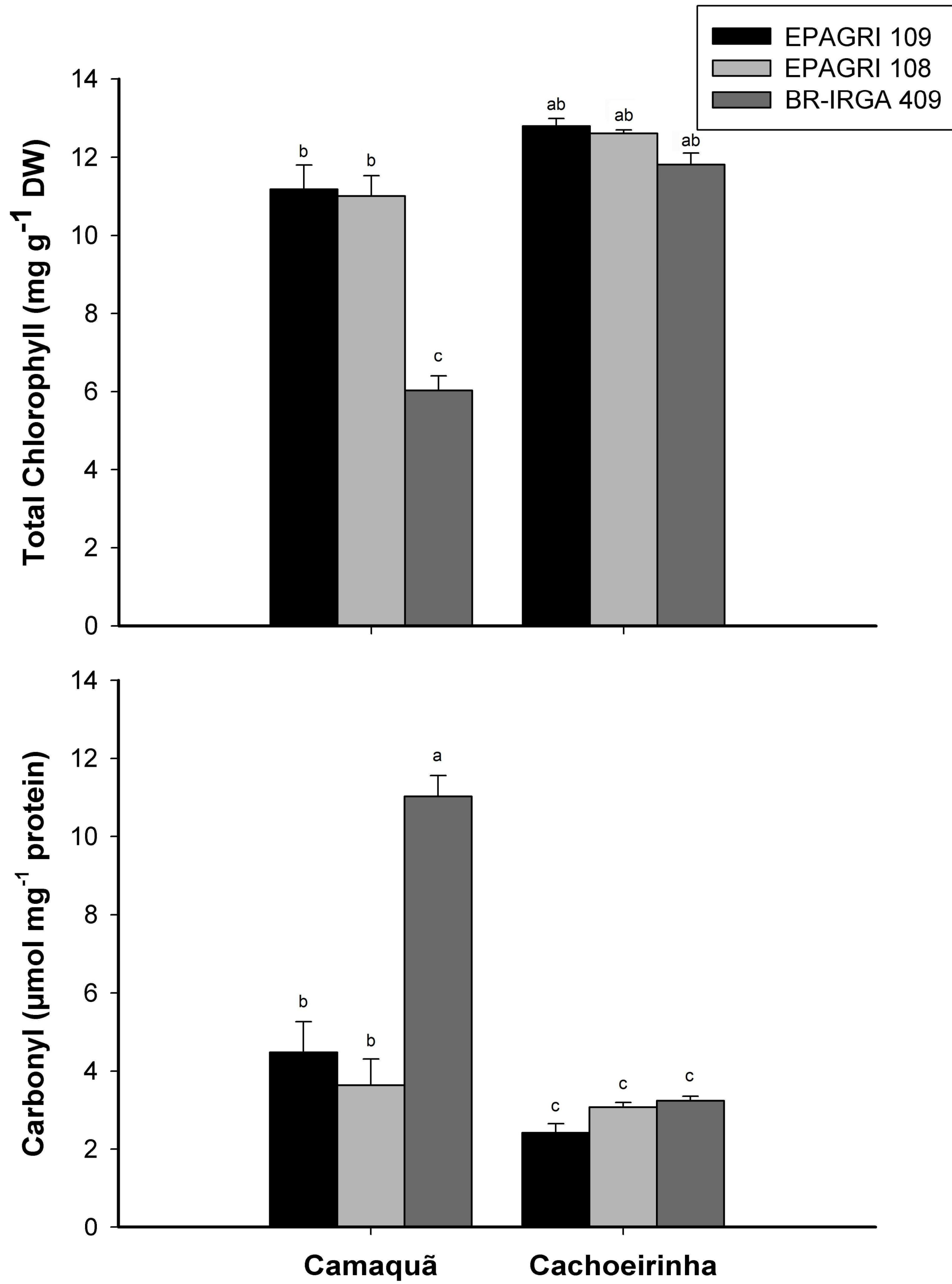


Figure 2

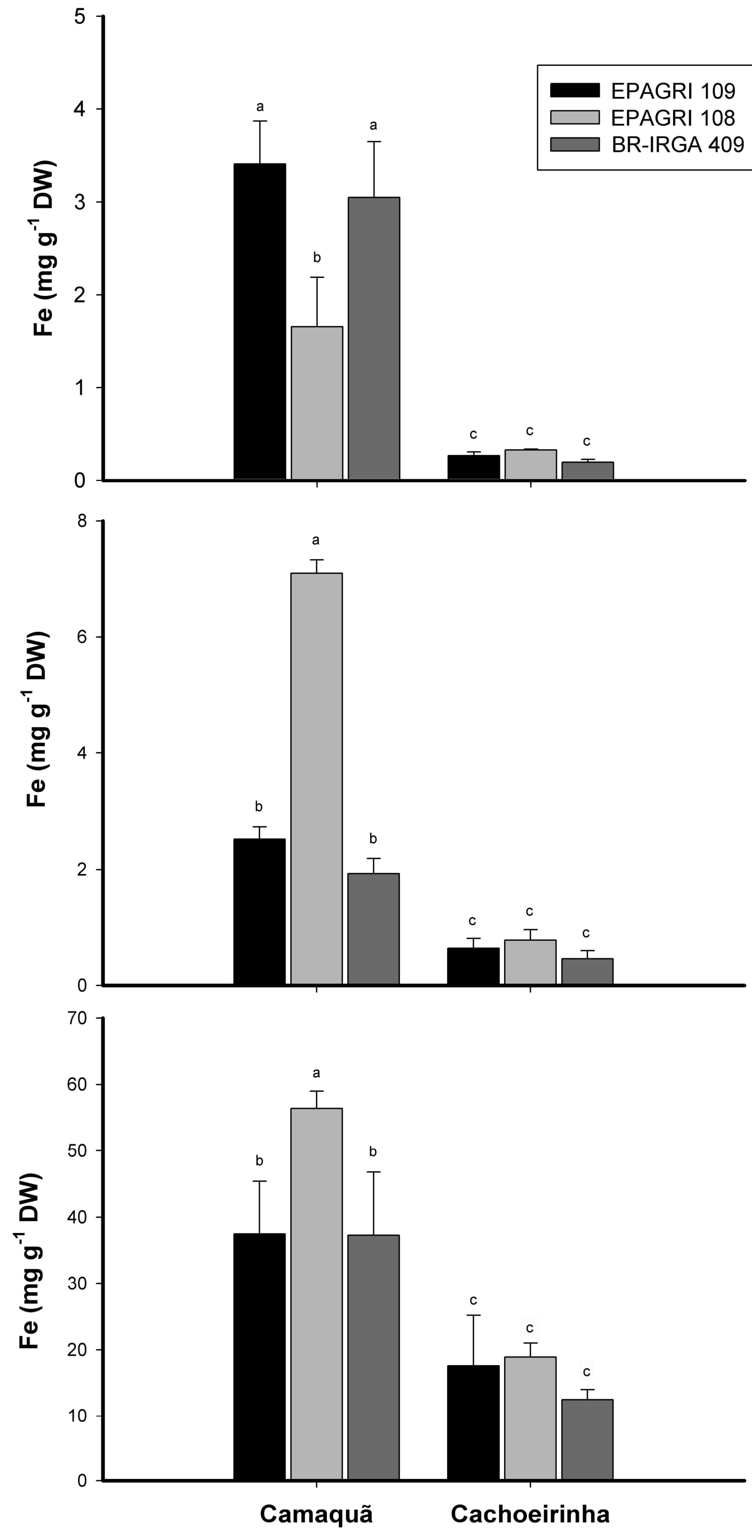


Figure 3

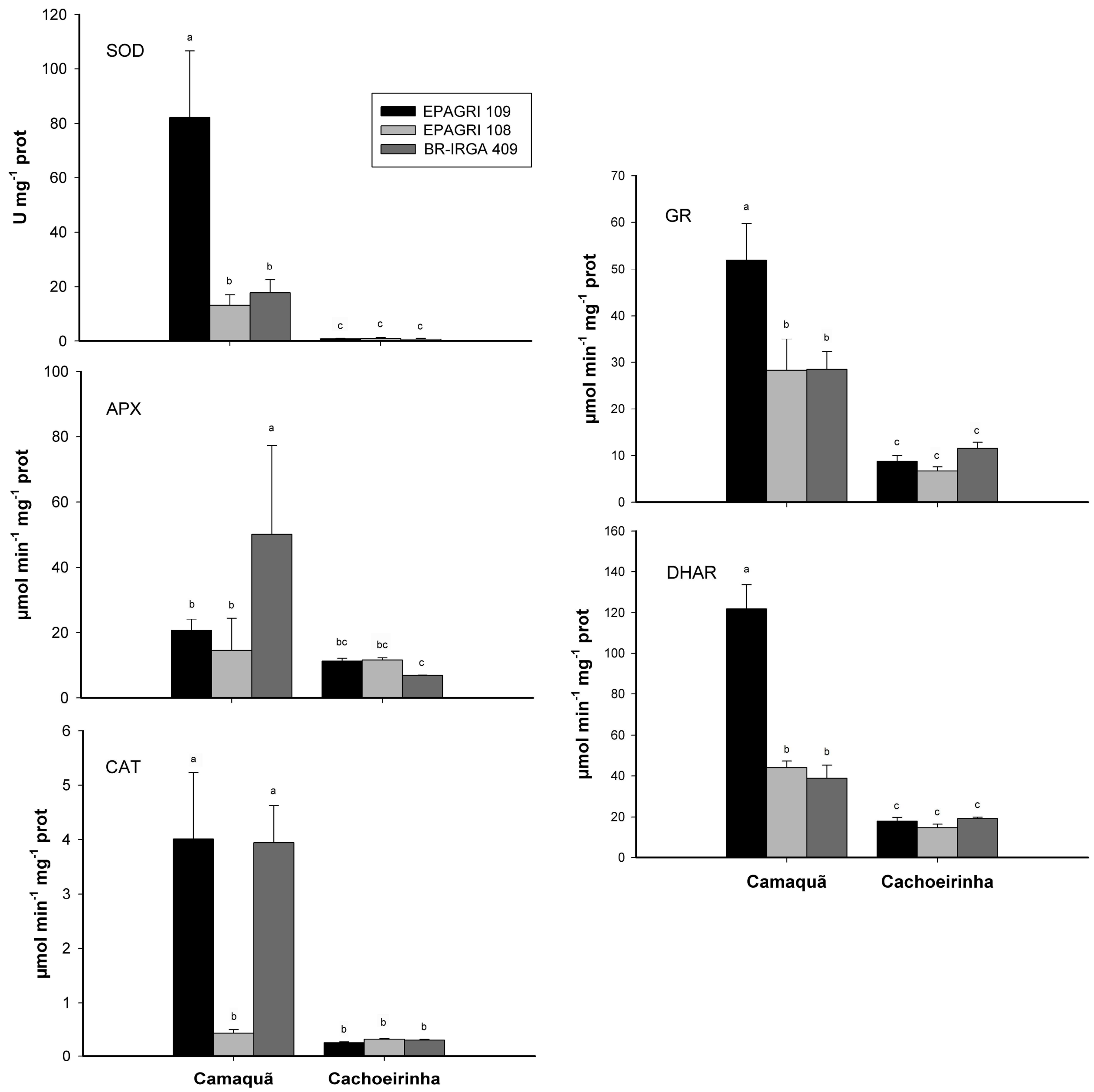
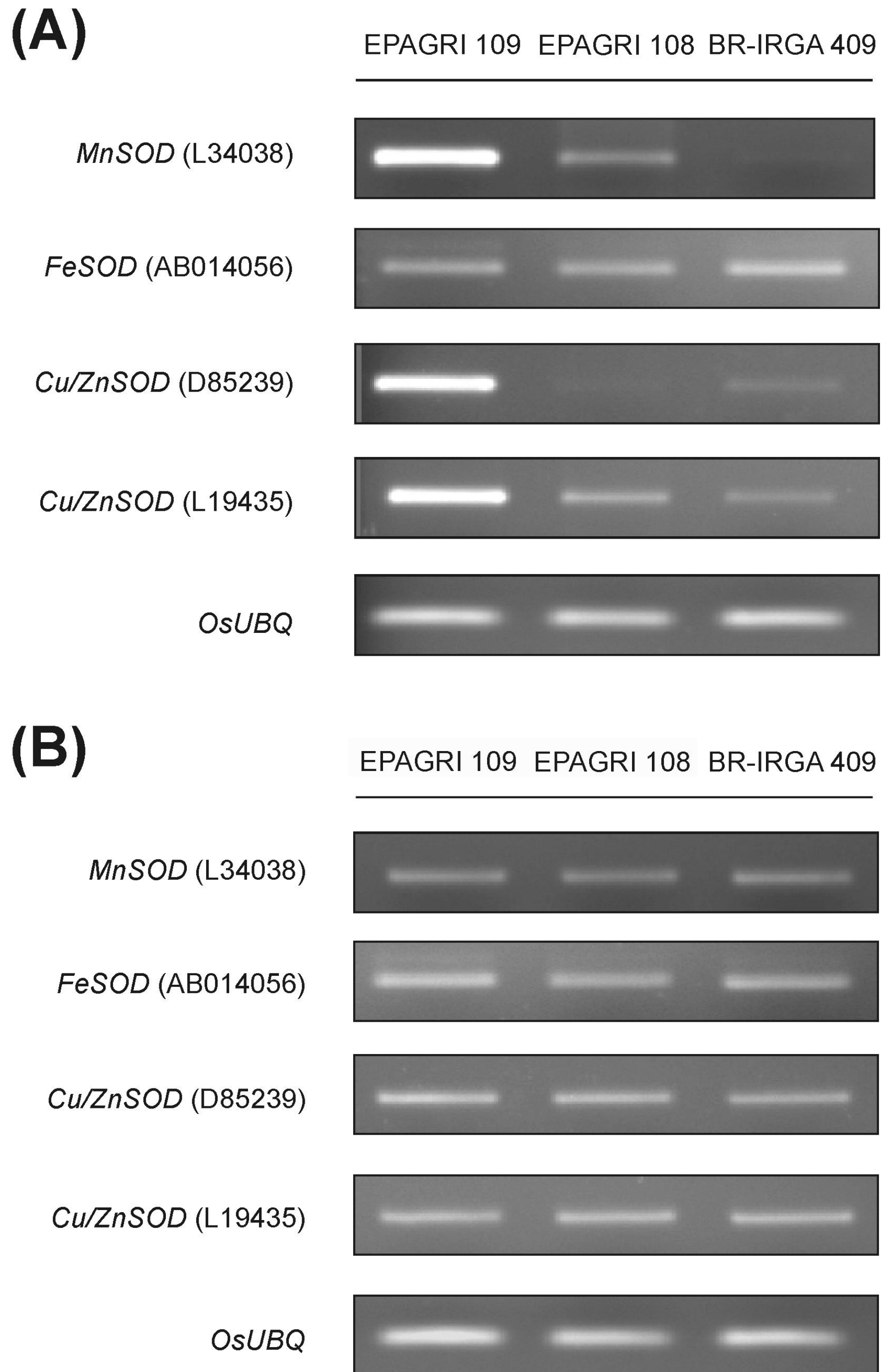


Figure 4



Capítulo 3

“Physiological assessment of excess iron toxicity and tolerance in rice”

Short running title: **Iron toxicity and tolerance in rice**

Physiological and molecular assessment of excess iron toxicity and tolerance in rice

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Abstract

Iron is an essential nutrient for plants. However, when in excess, it can generate oxidative stress. Iron toxicity is a major nutritional disorder in rice, and rice genotypes differ widely in tolerance to iron toxicity. To investigate the physiological basis of iron toxicity and tolerance, we analyzed the effects of iron excess on growth, photosynthetic activity, antioxidative metabolism and on the gene expression profile of two rice cultivars with distinct tolerance to iron toxicity (EPAGRI 108 – tolerant; BR-IRGA 409 – susceptible). After nine days of exposure to iron excess BR-IRGA 409 plants developed typical symptoms of iron toxicity and showed decreased biomass and chlorophyll content. The exposure to high levels of iron had a dramatic impact on photosynthesis, affecting the maximum carboxylation rate, electron transport rate and maximum apparent quantum yield, especially in the susceptible cultivar, and this toxic effect had a direct correlation with iron concentration and leaf oxidative damage. EPAGRI 108 plants accumulated lower amounts of iron than BR-IRGA 409 plants in both roots and shoots. Plants from the susceptible cultivar also showed higher levels of APX activity, probably as a response to oxidative damage. These results suggest that the tolerance to iron excess in EPAGRI 108 plants could not be explained by higher iron precipitation in the root apoplast and/or the leaf tissue tolerance mechanisms. Both cultivars also greatly differ in their root expression profile (seen by microarray analysis). While plants from the susceptible cultivar induced the expression of several genes involved in responses to stress (and down-regulated the expression of genes involved in iron uptake), plants from the tolerant cultivar induced the expression of several genes involved in cell wall biosynthesis and lignification (such as dirigent-like proteins). Therefore, tolerance mechanisms to iron excess could involve the capacity to avoid excessive iron uptake, with root lignification, limiting its uptake.

Introduction

Iron is an essential nutrient for virtually all living organisms. Due to its transition capability, it is involved in oxi-reductive reactions, like photosynthesis, respiration and nitrogen assimilation, among other important plant biological processes. When free and in excess inside the cell, iron can act as a potent generator of reactive oxygen species (ROS), specially the hydroxyl radical, through the Fenton reaction (Becana *et al.*, 1998). This radical is extremely toxic to cell metabolism, leading to oxidation of biological macromolecules like lipids, proteins and nucleic acids, causing membrane leaking and even cell death (Cadenas, 1989; Halliwell & Gutteridge, 1990). Iron excess causes oxidative stress in *Nicotiana plumbaginifolia* (Kampfenkel *et al.*, 1995) and in rice detached leaves (Yamauchi & Peng, 1995; Fang *et al.*, 2001). Excess iron can also increase the photodynamic action of cytochrome b_6/f affecting photosynthesis through these iron-dependent photosensitized reactions (Suh *et al.*, 2002). However, few data about the physiological implications of iron toxicity in whole plant systems is available.

In acid sulphate soils, high amounts of reduced iron (Fe^{2+}) become available and soluble due to the anoxic and reductive environment created by the waterlogging conditions (Ponnamperuma, 1972). Wetland rice stands for most of the world rice production. Iron toxicity reduces rice yield by 12 to 100%, depending on the genotype, intensity of iron toxicity stress and soil fertility status (Sahrawat, 2004). The occurrence of iron toxicity in rice plants is associated with high concentration of Fe^{2+} in the soil solution (Ponnamperuma *et al.*, 1955). Two distinct types of toxicity have been described in the literature: a true (or real) iron toxicity – characterized by the accumulation of toxic levels of iron in the plant body (Sahrawat, 2000; Olaleye *et al.*, 2001) and an indirect toxicity – a multiple nutritional

disorder, caused by deficiency of other nutrients like Ca, Mg and Zn (Benckiser *et al.*, 1984). Thus, the management of these two types of iron toxicities would require different strategies. The most cost-effective approach is the use of iron toxicity tolerant rice cultivars (Winslow *et al.*, 1989; Sahrawat *et al.*, 1996). The involvement of the root iron-exclusion power (or iron oxidation capacity) has been indicated as a possible mechanism used by rice cultivars to tolerate high amounts of iron in the soil solution (Ando *et al.*, 1983; Green & Etherrington, 1977), forming iron precipitates in the root's apoplast, generating the "iron plaque" (Bacha & Hossner, 1977). Leaf tissue tolerance to high levels of iron has been described as another mechanism involved in tolerance to iron toxicity, through the activity of cellular antioxidative defenses, involved in the metabolism of ROS (Wu *et al.*, 1998; Yamauchi & Peng, 1995). Using a hydroponic culture system, we analyzed the effects of high iron concentration on growth, photosynthetic activity and the gene expression profile of two rice cultivars, and discuss possible mechanisms involved in toxicity and tolerance to iron excess.

Materials and methods

Plant material, growth and treatments

Seeds from rice (*Oryza sativa* L. ssp. *indica*) cultivars EPAGRI 108 and BR-IRGA 409 (tolerant and susceptible to iron toxicity, respectively) were surface sterilized in ethanol 70% for two minutes, followed by $NaClO_4$ 1.5% for one minute. Seeds were washed with abundant distilled water and germinated on moistened filter paper in Petry dishes. The seedlings were kept in the dark during the first 48 hours, transferred to 16h/8h day light regime at 28°C for two days and then transferred to pots with vermiculite, watered with

nutrient solution (Yoshida, 1981), using $FeCl_3$ as the iron source. After 10 days, plants were transferred to hydroponic conditions, using the same nutrient solution, and after 10 more days plants were subjected to iron excess (500 ppm of iron) and control concentration (6.5 ppm), both using $FeSO_4$ as the iron source. To avoid possible effects of sulfur concentrations, Na_2SO_4 was added in the control treatment in the same equimolar concentration than in the iron excess treatment. To maintain the concentration and keep iron soluble, nutrient solutions were replaced every 72 hours.

Dry weight and chlorophyll determinations

After nine days of exposure to treatments, plants were separated in shoots and roots, immediately frozen in liquid nitrogen and kept at –20°C until further analysis.

Shoots and roots were dried at 60°C to constant weight for the determination of dry weight (DW). Fully expanded leaves were ground in liquid nitrogen and chlorophyll extracted in acetone 85%. Total chlorophyll (chlorophyll *a* + chlorophyll *b*) was quantified by measuring absorbance at 663 nm and 645 nm, and the concentrations calculated according to Ross (1974).

Gas exchange measurements

Gas exchange measurements were performed after one, two, three, six and nine days of exposure to the iron treatments, using a portable photosynthesis system (LI-6400, LiCor Inc., Lincoln, NE, USA). All determinations of photosynthetic rate were performed using a reference CO_2 concentration of 400 $\mu L\ L^{-1}$, 1000 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux (PPF) and leaf temperature of 22°C, using only the youngest fully expanded leaf. Light

response curves were performed using a reference CO_2 concentration of 400 µL L^{-1}, leaf temperature of 22°C and a PPF range from 1500 to 0 µmol $m^{-2}s^{-1}$. CO_2 response curves were performed using 1000 µmol m^{-2} s^{-1} PPF, leaf temperature of 22°C, with reference CO_2 concentrations ranging from 800 to 50 µL L^{-1}. All photosynthetic response curves were performed using only the youngest fully expanded leaf, after six days of exposure of plants to treatments (control and iron excess), and the photosynthetic parameters were estimated according to the biochemical model described in Farquhar *et al*. (1980).

Iron content determinations in shoots, roots and iron plaque

To determine the iron content in the iron plaque, root systems were washed in abundant distilled water and immediately kept for 3 hours in cold DCB (dithionite-citrate-bicarbonate) solution (Taylor & Crowder, 1983) and iron content determined by atomic absorption spectrophotometry (Varian-Model Spectra 10/20, Victoria, AU). After extraction of iron plaque, root systems were washed in distilled water and dried at 60°C. Dry samples (shoots and roots) were ashed at 500°C for 3 hours, the ashes were digested with concentrated HCl and iron was quantified by atomic absorption spectrophotometry.

Oxidative Damage to Lipids, Proteins and H_2O_2 determination

Lipid peroxides were extracted in ethanol 80% from fully expanded leaves and lipid peroxidation determined by measuring the concentration of thiobarbituric acid-reacting-substances (TBARS) as described by Hodges *et al*. (1999). Oxidative damage to proteins was determined by the quantification of carbonyl groups, by derivatization with 2,4-dinitrophenyl-hydrazine. Fully expanded leaves were ground in cold extraction buffer (50

mM Tris (pH 8.0), 2 mM EDTA, 1 mM PMSF and 1 mM benzamidine), centrifuged at 12.000 x g for 15 min at 4°C, and the supernatants immediately used for carbonyl determination according to Levine *et al*. (1990), and normalized with the protein content determined using the dye-binding method (Bradford, 1976). Hydrogen peroxide was quantified spectrophotometrically (Cintra 5, GBC Scientific Equipment, Victoria, AU) after extraction with 0.1% TCA and reaction with KI in the dark (Alexieva *et al.*, 2001). The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentrations.

Antioxidant enzymes activity

For all enzymatic activity determinations, fully expanded leaves were ground in cold extraction buffer containing 50 mM of sodium phosphate buffer (pH 7.4), 1% PVP, 1 mM EDTA, 1mM PMSF and 1mM benzamidine. The homogenate was centrifuged at 12000 g for 15 min at 4°C and the supernatant immediately used for enzymatic assays.

Ascorbate peroxidase (APX) activity was determined according to Klapheck *et al.* (1990), from the decrease in absorbance at 290 nm, catalase (CAT) activity was determined following the decrease of absorbance at 240 nm due to H_2O_2 consumption (Cakmak & Marschner, 1992) and superoxide dismutase (SOD) activity was quantified as described by Beyer and Fridovich (1987), using 15 min of illumination and recording the absorbance at 560 nm. All enzymatic assays were performed at 25°C as initial activities, with no lag period, and protein determined by the dye-binding method (Bradford, 1976).

Microarray analysis

For the microarray analysis, highly purified total RNA was isolated using NucleoSpin RNA II (Macherey-Nagel) from roots from the studied cultivars (EPAGRI 108 and BR-IRGA 409) exposed for three days to control or iron excess treatments. RNA purity and quality was assessed by absorbance (at 260 and 280 nm) and by the Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analysis was performed using the GeneChip Rice Genome Array (Affymetrix), containing probes to query 51,279 transcripts representing two rice subspecies, with approximately 48,564 japonica transcripts and 1,260 transcripts representing the *indica* subspecies. The microarray analysis was performed using one-cycle target labeling and control reagents (Affymetrix) using 5 μg of total RNA as starting material for each sample. Target preparation, hybridization to arrays, washing, staining, and scanning, were carried out according to manufacturer's instructions (Affymetrix). Affymetrix GeneChip Operating Software 1.2.1 was used for washing and scanning in the Fluidics Station 450 (Affymetrix) and the Scanner 3300 (Affymetrix), respectively. Sample quality was assessed by examination of 3' to 5' intensity ratios of Poly-A controls, hybridization controls, and housekeeping genes. For further data analysis, the probe intensity files (.cel) were imported into ArrayAssist software (Stratagene) and the normalization and probe summarization was performed using the Robust Multichip Analysis (RMA) algorithm (Irizarry *et al*., 2003) by the probe logarithmic intensity error method, followed by variance stabilization. The variance stabilization step stabilizes the variance across the entire range of expression, including the genes with low expression.

To identify differentially expressed genes, a student's t test (using $P \leq 0.05$) was performed and the genes that were up- or down-regulated by 3-fold or more were considered to be

significantly differentially expressed. The average from three biological replicates for each sample was used for analysis.

Statistical Analysis

Means from physiological and biochemical analyses were compared by analysis of variance (ANOVA), followed by Duncan test, according to Quinn & Keough (2002). Differences were considered significant when $P \leq 0.05$.

Results

Growth and chlorophyll content

After nine days of exposure to iron excess, plants from the susceptible cultivar, BR-IRGA 409, developed typical symptoms of iron toxicity, with the appearance of bronzing and necrotic lesions on the leaves, and roots acquired a dark brown and orange color (Figure 1). These symptoms came associated with a decrease in shoot and root dry weight (Figure 1e and Figure 1f) in the BR-IRGA 409 cultivar, and no significant differences were observed in shoot and root dry weight from the tolerant cultivar (EPAGRI 108). Exposure to iron excess also led to a significant decrease in total chlorophyll content in both cultivars, mostly in the susceptible cultivar (Figure. 1g).

Gas exchange measurements

After nine days of exposure to iron excess, the susceptible cultivar (BR-IRGA 409) showed lower rates of light saturated photosynthesis when compared to control plants. On the other hand, after the same period of time, EPAGRI 108 plants showed no significant difference

between treatments (Figure 2). The negative effect on carbon assimilation initiated after the first day of exposure to iron excess and increased with time in BR-IRGA 409. Interestingly, after the first 24 hours of exposure, the tolerant cultivar had a similar decrease in photosynthetic activity. However, after this initial drop, there was a recovery of the photosynthetic activity in EPAGRI 108 plants, and after six days of exposure to iron excess the photosynthetic activity reached levels similar to those of the control treatment.

Different patterns for the A/Ci (CO_2 assimilation rates / estimated substomatal CO_2 partial pressure) response curves were observed in EPAGRI 108 and BR-IRGA 409 plants (Figure 3A and B). Under iron excess, plants from both cultivars showed a reduction in photosynthetic capacity (the maximum rate of photosynthesis reached under CO_2 saturation as light is already saturating), which was much more evident in BR-IRGA 409 plants. In this cultivar, there was also a decrease in the slope of the A/Ci relationship, indicating a reduction in the carboxylation efficiency. EPAGRI 108 plants, however, had no reduction on the same slope, indicating no effects of the treatment on carboxylation efficiency. Analysis of the response curves through the model of Farquhar *et al.* (1980) indicated that iron excess led to a decrease in maximum carboxylation rate ($Vc_{max,}$, 24.5% reduction) and electron transport rate (J_{max}, 41.7% reduction) after six days of exposure only in the susceptible cultivar (BR-IRGA 409), compared to the control treatment (Table 1). EPAGRI 108 had a slight increase in both values, although not statistically significant.

The light response curves (Figure 3C and D) revealed a clear advantage of BR-IRGA 409 under non stressful conditions, while EPAGRI 108 responded similarly to both control and excess iron treatments, through the whole range of light levels. Exposure of BR-IRGA 409 plants to iron excess resulted in a significant reduction (51.9%) on apparent quantum yield

(ϕ_m) when compared to control plants, while a small reduction (6.3%, not significant) was seen in EPAGRI 108 plants (Table 1).

Iron accumulation and distribution

In both cultivars, exposure to iron excess led to an increase in iron content in shoots and roots (Table 2). This increase was more pronounced in plants from the susceptible cultivar. Shoots from BR-IRGA 409 plants submitted to iron excess showed a seven-fold increase in iron content, compared to a four-fold increase in EPAGRI 108 plants. The roots maintained a similar pattern, with a three-fold increase in BR-IRGA 409 and a 1.3-fold increase in EPAGRI 108. The iron content in the root apoplast (as "iron plaque") was higher in plants submitted to iron excess treatment than in control plants (Table 2), increasing 85% in EPAGRI 108 and 166% in BR-IRGA 409 plants.

Oxidative damage to lipids, proteins and H_2O_2 accumulation in leaves

Fully expanded leaves from BR-IRGA 409 plants showed higher TBARS and carbonyl concentration, and also higher H_2O_2 levels, while no difference could be observed in EPAGRI 108 (Figure 4). These data clearly confirmed that the iron excess treatment caused oxidative stress only in the susceptible cultivar (BR-IRGA 409), leading to the oxidation of lipids, proteins and the H_2O_2 accumulation in the leaf tissue.

Antioxidative enzymes activity (CAT, APX and SOD)

The activities of antioxidative enzymes changed differently with the exposure to iron excess (Figure 5). Treatment with 500 ppm of iron resulted in an increased CAT and APX

activities in both cultivars, but the highest increment in APX activity was observed in fully expanded leaves from BR-IRGA 409. No difference in SOD activity was observed in plants submitted to iron excess (Figure 5).

Gene expression profile in plants exposed to iron excess

Total RNA samples were purified from roots of EPAGRI 108 and BR-IRGA 409 plants which had been exposed to the treatments for three days and their gene expression profile was analyzed using the RiceGene Chip genome array (Affymetrix). As expected, exposure to high iron concentration distinctly affected the expression profile of the two studied cultivars (Figure 6). There was alteration in the expression profile of 520 genes (428 up-regulated and 92 down-regulated genes) in the susceptible cultivar (BR-IRGA 409) and of 353 genes in the tolerant cultivar (43 up-regulated and 310 down-regulated genes).

Among the up-regulated genes found in BR-IRGA 409 (Table 3), eleven genes involved in metal transport or homeostasis were found, such as CC1 (a gene similar to the *Arabidopsis* vacuolar iron transporter1- *AtVIT1*), two metallothioneins (*OsMT1* and *OsMT3a*), the two rice ferritin genes (*OsFER1* and *OsFER2*), *OsZIP5* (a Zn^{2+} transporter), one nicotianamine synthase isoform (*OsNAS3*), and other metal ion binding proteins (Table 3). We also found several stress-related genes up-regulated in this cultivar, such as two ABA-stress-ripening proteins (*OsASR3* and *OsASR1*), two heat shock proteins (17.5 and 17.4 kDa heat shock proteins), six peroxidases (*OsPrx104*, *OsPrx110*, *OsPrx135*, *OsPrx46*, *OsPrx57* and *OsPrx93*), among other up-regulated genes (such as nine nucleotide-binding proteins – four WRKY, three MYB and two NAC transcriptions factors).

In the same cultivar, iron excess led to down-regulation of several genes directly or putatively involved in iron uptake, such as *OsYS7*, *OsYS6*, *OsYS14*, *OsIRT1* and

OsNRAMP1 (Table 4) and three genes involved in the biosynthesis of nicotianamine were down-regulated (*OsNAS1*, *OsNAS2* and *OsNAAT1*). Also, seven peroxidases (*OsPrx95*, *OsPrx78*, *OsPrx41*, *OsPrx20*, *OsPrx12*, *OsPrx107* and *OsPrx95*) and three Dirigent-like protein were down-regulated by the exposure to iron excess in BR-IRGA 409, among other genes.

In contrast, in EPAGRI 108 iron excess led to the up-regulation of four genes involved in cell wall lignification (three Dirigent-like proteins and one laccase), two genes involved in lignin biosynthesis (a caffeoyl-CoA *O*-methyltransferase and a *O*-methyltransferase ZR4) and also genes involved in cell wall biosynthesis (such as the three xyloglucan endotransglucosylase/hydrolase – *OsXTH6*, *OsXTH10* and *OsXTH18*), among other genes involved in the response to abiotic/biotic stresses (such as four peroxidases – *OsPrx78*, *OsPrx20*, *OsPrx20* and *OsPrx41*) and with unknown function (Table 5). Among the down-regulated genes found in EPAGRI 108, we found several genes involved in abiotic/biotic stress responses, such as five xylanase inhibitor proteins, two ABA-stress-ripening genes (*OsAsr3* and *OsAsr4*), *OsAOX1b* and six peroxidases (*OsPrx57, OsPrx46, OsPrx135, OsPrx131, OsPrx111* and *OsPrx110*) (Table 6). Iron excess down-regulated the expression of several nucleotide binding proteins, as well genes with unknown function.

Discussion

Iron excess led to decreased biomass accumulation (in shoots and roots), loss of chlorophyll and decreased photosynthetic activity in the susceptible cultivar. The toxic effects observed were directly correlated with accumulation of higher levels of iron in shoots and roots of BR-IRGA 409 plants. Olaleye *et al*. (2001) also reported that high concentrations of iron

resulted in decreased plant weight and grain yield, and the growth retardation observed was not attributed to deficiencies of other nutrients, but to physiological problems directly originated from excessive iron accumulation. Working with the same rice cultivars used in our experiments, Silveira *et al*. (2007) showed that the toxic effects of exposure to iron excess did not induce deficiency of other nutrients, indicating a direct effect of iron in its toxicity. The decrease in chlorophyll content could be a result of oxidative damage derived from excessive accumulation of iron in the leaf tissue. Similar results were observed in sunflower leaves exposed to iron, copper and cadmium (Gallego *et al.*, 1996). This decrease could lead to decreased light capture through the photosystem II (PSII) antennae, resulting in reduced apparent quantum yield, as seen in our results.

In this study, photosynthetic activity decreased in plants from both cultivars after the initial period of exposure to iron excess. However, the tolerant cultivar (EPAGRI 108) was able to fully recover its photosynthetic capacity after six days, a reasonable lag period for the induction of its tolerance mechanisms. Using A/Ci and light response curves we could identify the impact of iron in carbon fixation, affecting the maximum carboxylation rate (Vc_{max}), electron transport rate (J_{max}) and maximum apparent quantum yield (ϕ_m) in the susceptible cultivar. The reduction in Vc_{max} may result from the reduced rate of electron transport, limiting the amount of available energy for Calvin cycle enzymes. The toxic effects of iron in photosynthesis were also observed in *N. plumbaginifolia* cuttings accompanied by photoinhibition, increased reduction of PSII and higher thylakoid energization (Kampfenkel *et al.*, 1995). The reduction in chlorophyll content associated to lower electron transport rates may indicate a direct effect of excess iron on the photosynthetic electron transport chain components (either LHCII or $Cytb_6/f$). Excessive

amounts of iron in thylakoid membranes in the form of cytochrome b_6/f complex were correlated with photodamage to PSII, derived from excessive production of singlet oxygen in pea plants (Suh *et al.*, 2002).

Iron toxic effects had a direct correlation with its accumulation in the plant body. Iron content in shoots and roots of plants exposed to iron excess from both cultivars were above the critical level for iron toxicity (0.3-0.5 mg g^{-1} DW) (Foy *et al.*, 1978; Yoshida, 1981), with BR-IRGA 409 plants showing significantly higher accumulation of iron in the plant body than EPAGRI 108 plants. One possible mechanism involved in tolerance to iron toxicity used by EPAGRI 108 could be root exclusion of iron, limiting the absorption of iron through the root system. In fact, the root capacity to oxidize iron has been described as a possible mechanism of tolerance (Ando *et al.*, 1983; Green & Etherrington, 1977). However, the susceptible cultivar (BR-IRGA 409) had significantly higher levels of iron in the apoplast of the root system, forming the so-called “iron plaque” (or iron coating). After nine days of exposure to iron excess both cultivars accumulated higher levels of iron in the iron plaque, but no relationship between tolerance to iron excess and iron plaque could be observed, indicating that the capacity of the root system to oxidize iron was not involved in the mechanism of tolerance used by EPAGRI 108 plants.

The lower iron accumulation in EPAGRI 108 than in BR-IRGA 409 plants could be related to the distinct metabolic profiles of both cultivars. In the control treatment, BR-IRGA 409 plants showed higher assimilation rates, higher biomass accumulation and higher transpiration rates (data not shown) than EPAGRI 108 plants. Lower metabolic rates and lower mass flow in the transpiration stream could have an impact on lower iron accumulation in these plants. However, iron concentrations were very similar in both cultivars when submitted to the control treatment, and became higher in BR-IRGA 409

plants only in the iron overload treatment, the condition where this same cultivar is the one mostly affected, with major reductions in biomass, carbon assimilation and transpiration rates.

The activities of CAT and APX were significantly higher in plants exposed to iron excess, and APX was remarkably higher in BR-IRGA 409. Both enzymes are involved in the detoxification of H_2O_2 in plant cells, and removal of H_2O_2 from the cytosol, chloroplast and mitochondria of higher plants is generally attributed to APX, while CAT is mainly localized in peroxisomes and in the cytosol (Asada, 1992; Jimenez *et al.*, 1997). The involvement of CAT in response to environmental stresses has been described as a consequence of increased photorespiration (Foyer *et al.*, 1994). High photorespiration rates may divert the electron flow under excessive light, thereby protecting the photosynthetic apparatus and especially the PSII, attenuating ROS production in the chloroplast (Noctor *et al.*, 2002)**.** Under stressful conditions, the generation of catalytic iron (free iron, capable of catalyzing free-radical generation) and its accumulation is correlated with oxidative damage (Moran *et al.*, 1994). Possibly, the H_2O_2 generated though photorespiration could react with excess iron to generate oxygen radicals through a Fenton type reaction (Schützendübel & Polle, 2002). Since BR-IRGA 409 plants accumulate higher amounts of iron in shoots, the toxic effects observed could be a direct result of increased ROS production, derived from the Fenton reaction. Despite the contribution of CAT and APX in detoxifying the oxidative stress generated by iron excess, no relation between their activity and tolerance in EPAGRI 108 plants could be observed. The higher activity of APX observed in BR-IRGA 409 plants exposed to iron excess could be related to the higher content of H_2O_2 observed in leaves from the same cultivar, possibly as a consequence of its higher iron levels.

It is clear that the capacity to decrease iron uptake greatly contributes to the tolerance character of EPAGRI 108. Several genes involved in lignification (such as the Dirigent-like proteins) and cell wall biosynthesis were up-regulated in roots exposed to iron excess in this cultivar. Dirigent proteins stipulate, at regional and stereochemical levels, the outcome of coupling of two molecules of *E*-coniferyl alcohol to produce the lignan (+)-pinoresinol (Davin *et al*., 1997), being expressed mainly in lignifying tissues (Burlat *et al*., 2001). This protein requires the provision of one-electron oxidation through an auxiliary source (eg. laccases, peroxidases, monoxigenases) for its activity (Burlat *et al.*, 2001). Indeed, iron excess induced the expression of a laccase in the same cultivar, a protein that belongs to a class of enzymes known to be associated with the oxidation of *o*- and *p*-quinols, participating in the lignin biosynthesis (Dean *et al*., 1998) and also highly expressed in lignifying tissues (Sato *et al*., 2001). In the same cultivar, iron induced the expression of four peroxidases (*OsPrx78*, *OsPrx20*, *OsPrx107* and *OsPrx41*), which also could be responsible for the oxidation required for lignin biosynthesis. However, iron excess also induced the expression of several peroxidases in the susceptible cultivar (*OsPrx104*, *OsPrx110*, *OsPrx135*, *OsPrx46*, *OsPrx57* and *OsPrx93*). Peroxidases are heme-containing proteins that catalyze the reduction of H_2O_2 by taking electrons to various donor molecules (e.g. phenolics, lignin precursors or secondary metabolites). The diversity of the reactions catalyzed by plant peroxidases explains the implication of these proteins in a broad range of physiological processes, such as auxin metabolism, lignin and suberin formation, cross linking of cell wall components, defense against pathogens, cell elongation and protection against oxidative stress (Penel *et al*., 1992; Hiraga *et al*., 2001). In the rice genome, 138 peroxidase genes were described (Passardi *et al*., 2004), and the exact physiological function displayed by each peroxidase isoform is unknown. Different peroxidase isoforms,

probably with different physiological roles, were found to be up-regulated by iron excess in the two rice cultivars tested in our study.

Iron excess also led to the up-regulation of three xyloglucan endotransglucosylase/hydrolases (*OsXTH6*, *OsXTH10* and *OsXTH18*), proteins belonging to a family of enzymes that specifically use xyloglucan as a substrate, and catalyse xyloglucan endotransglucosylase and/or xyloglucan endohydrolase activities (Yokoyama *et al.*, 2004), being thought to play an important role in the construction and restructuring of xyloglucan cross-links, and induced the expression of two glycine-rich proteins. Glycine-rich proteins are characterized by their repetitive primary structure, which contains up to 70% glycine arranged in short amino acid repeat units (Condit & Meagher, 1986). A close functional relationship between glycine-rich proteins and lignin deposition during cell wall biogenesis was suggest (Keller, 1988), and have been clearly associated with cells that are destined to be lignified (Showalter, 1993). Along with this, two genes involved in lignin biosynthesis, a caffeoyl-CoA *O*-methyltransferase and a *O*-methyltransferase ZR4 (Boerjan *et al.*, 2003) were up-regulated in EPAGRI 108. Taken together, our results indicated that the lignification and remodeling of the cell wall could be an important feature in the tolerance character displayed by EPAGRI 108 plants. The remodeling of the cell wall was also indicated as an important feature in tolerance to Al in rice (Yang *et al.*, 2007), indicating that changes in the chemical composition of the root cell wall have a great importance against stressful conditions.

As expected, iron excess induced the expression of several genes involved in responses to abiotic and biotic stresses in the susceptible cultivar (BR-IRGA 409), such as two ABA-stress-ripening proteins, *OsAsr1* and *OsAsr3*. The Asr (*A*BA-*s*tress-*r*ipening) proteins were correlated with responses to several abiotic stresses (Vaidyanathan *et al.*, 1999), acting as

non-histone chromosomal proteins involved in protection against a range of stress signals by modulating cell sugar traffic (Carrari *et al*., 2004). Iron promoted the expression of *OsAOX1b*, another gene known to be responsive to several abiotic stresses in rice (Ohtsu *et al*., 2002), and although the benefit remains uncertain, it may enhance the ability to resists to stressful conditions, through reducing the level of oxidative stress (Maxwell *et al*., 1999). Iron excess also induced the expression of several genes involved in metal transport and homeostasis. Among them, a gene coding a CC1 protein, with great similarity with *AtVIT1*, appears of a particular interest. In *Arabidopsis*, *AtVIT1* functions as a vacuolar iron transport, being responsible for the vacuolar iron storage in seeds (Kim *et al*., 2006). Whether this specific CC1 protein corresponds to a vacuolar iron transporter remains to be elucidated, but the accumulation of excessive amounts of iron in the vacuole is known as a possible protection mechanism against iron-mediated oxidative stress (Becker *et al*., 1998). Ferritins appear as important players against iron-mediated oxidative damage (Ravet *et al*., 2008). In rice, two ferritin genes were described (Gross *et al*., 2003), and both were up-regulated upon iron excess in our microarray experiment. Ferritins are holoproteins composed of 24 subunits that can accumulate up to 4500 iron atoms and its accumulation upon iron overload is known in several plant species (Briat *et al*., 1999).

Iron excess down-regulated the expression of several genes or related to its uptake, such as three genes belonging to the Yellow Stripe-Like gene family, transporters involved in the transport of metal-nicotianamine complexes (Curie e*t al*., 2009), and of genes involved in the biosynthesis of nicotianamine (such as *OsNAS1, OsNAS2* and *OsNAAT1*), indicating a coordinated response against excessive levels of iron in the solution with the down-regulation of genes involved in its high affinity uptake.

Interestingly, several genes that were down-regulated upon iron excess in the tolerant cultivar were up-regulated in the susceptible one, and vice-versa. These results could indicate that in an initial phase both cultivars respond in a similar way, inducing the expression of genes involved in response to stress and other functions. After this common responses, while EPAGRI 108 down-regulates the expression of stress-related genes and induces the expression of genes involved in cell wall remodeling and lignin synthesis, BR-IRGA 409 plants appears to regulate the same genes in the opposite direction.

In conclusion, iron excess led to a decrease in dry weight, chlorophyll and photosynthetic activity, and an increase in oxidative damage and H_2O_2 content in the susceptible cultivar, BR-IRGA 409. Our results confirm, with physiological data, the tolerant character of the EPAGRI 108 cultivar, and we suggest that the cell wall remodeling and lignification contributes to thid important character of EPAGRI 108.

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Table 1. Effects of six-days exposure to control (Ctrl) or excess (Fe+) iron treatments on photosynthetic parameters of rice plants from cultivars EPAGRI 108 and BR-IRGA 409. Each value represents the mean of four replicates. Distinct letters indicate significant difference between means from each parameter (*P* ≤ 0.05). ϕ_m= apparent quantum yield; Vc_{max}= maximum carboxylation rate; J_{max}= electron transport rate.

	EPAGRI 108		BR-IRGA 409	
	Ctrl	Fe+	Ctrl	Fe+
ϕ_m (mol CO_2 mol^{-1} photons)	0.0369±0.0059b	0.0394±0.0044b	0.0524±0.0043a	0.0252±0.0056c
Vc_{max} (µmol CO_2 m^{-2}s^{-1})	17.75±2.05c	18.65±1.74c	30.78±0.7a	23.25±2.30b
J_{max} (µmol m^{-2} s^{-1})	58.3±15.27b	65.6±12.52b	111.55±12.59a	65.05±5.73b

Table 2. Iron accumulation and distribution in rice plants from cultivars EPAGRI 108 and BR-IRGA 409 after nine days of exposure to control (Ctrl) or excess (Fe+) iron treatments. Each value represents the mean of six replicates. Distinct letters indicate significant difference between means from each parameter ($P \leq 0.05$).

	EPAGRI 108		BR-IRGA 409	
	Ctrl	Fe+	Ctrl	Fe+
Shoot (mg Fe g^{-1} DW)	0.30±0.02c	1.25±0.11b	0.29±0.03c	2.04±0.21a
Root (mg Fe g^{-1} DW)	0.91±0.16c	1.97±0.23b	0.81±0.15c	2.58±0.2a
Iron plaque (mg Fe g^{-1} DW)	48.07±0.94d	88.81±2.53b	39.04±2.58c	104±2.65a

Table 3. Up-regulation of Fe-responsive genes in the iron-excess susceptible cultivar BR-IRGA 409.

Functional categories	Probe set ID	TIGR gene Locus	Fold change	*P* value
Metal Transport/Homeostasis				
CC1 protein (similar to *AtVIT1*)	Os.Affx.6372.1.S1_s_at	LOC_Os09g23300	37.279	0.000
OsZIP5 (Zn^{2+} transporter)	Os.54471.1.S1_at	LOC_Os08g10630	3.301	0.001
OsMT1 (metallothionein)	Os.Affx.32080.1.S1_at	LOC_Os12g38290	3.468	0.000
OsMT3a (metallothionein)	Os.7992.1.S1_a_at	LOC_Os01g10400	6.186	0.000
Metal ion transporter, putative	Os.5643.1.S1_at	LOC_Os01g64890	3.583	0.000
Copper ion binding protein	Os.23181.1.S1_at	LOC_Os01g03530	3.961	0.002
Metal tolerance protein C3	Os.18637.1.S1_at	LOC_Os02g53490	3.890	0.001
Metal ion binding protein	Os.24473.1.A1_s_at	LOC_Os11g03910	4.017	0.000
OsFER1 (ferritin)	Os.12096.4.S1_s_at	LOC_Os11g01530	3.852	0.000
OsFER2 (ferritin)	Os.12096.3.S1_a_at	LOC_Os12g01530	4.329	0.000
OsNAS3 (nicotianamine synthase)	Os.1479.1.S1_at	LOC_Os07g48980	4.471	0.000
Abiotic/Biotic stress related				
OsAsr3 (ABA, stress, ripening)	Os.27963.1.A1_at	LOC_Os01g72900	25.642	0.000
OsAsr1 (ABA, stress, ripening)	Os.12094.1.S1_a_at	LOC_Os02g33820	4.619	0.000
17.4 kDa class I heat shock protein	Os.37773.1.S1_at	LOC_Os03g16030	27.024	0.000
17.5 kDa class II heat shock protein	Os.519.1.S1_at	LOC_Os01g08860	9.736	0.002
ABA-responsive protein	Os.53117.1.S1_x_at	LOC_Os12g29400	18.904	0.000
Dehydration stress-induced protein	Os.6812.1.S1_at	LOC_Os10g21670	18.299	0.002
OsAOX1b (alternative oxidase)	Os.3406.1.S1_at	LOC_Os04g51160	11.972	0.000
Desiccation-related protein	Os.46073.1.A1_at	LOC_Os04g33150	10.582	0.000
OsPBZ1 (probenazole inducible gene)	Os.165.1.S1_at	LOC_Os12g36880	13.727	0.009
OsGSTU5 (glutathione S-transferase)	Os.49030.1.A1_s_at	LOC_Os09g20220	6.246	0.003
OsGSTU6 (glutathione S-transferase)	Os.4762.1.S1_at	LOC_Os10g38740	5.680	0.002
OsPrx104	Os.53161.1.S1_x_at	LOC_Os07g34710	8.668	0.000
OsPrx110	Os.2237.1.S1_at	LOC_Os07g48010	4.995	0.001
OsPrx135	Os.11549.1.S1_at	LOC_Os12g02080	8.155	0.000
OsPrx46	Os.2961.1.S1_at	LOC_Os03g25340	9.032	0.000
OsPrx57	Os.55918.1.S1_at	LOC_Os04g55740	6.665	0.000
OsPrx93	OsAffx.15978.1.S1_at	LOC_Os06g48000	6.603	0.001
Nucleotide-binding protein				
OsWRKY11	Os.30512.1.S1_at	LOC_Os01g43650	5.338	0.000
OsWRKY19	Os.55597.1.S1_at	LOC_Os05g49620	4.212	0.000
OsWRKY76	Os.25606.1.S1_at	LOC_Os09g25060	3.123	0.017
OsWRKY77	Os.30657.1.S1_at	LOC_Os01g40260	5.490	0.001
MYB transcription factor	Os.31381.1.S1_at	LOC_Os01g03720	3.257	0.000
MYB transcription factor	Os.20224.1.S1_at	LOC_Os12g37690	3.231	0.002
MYB-like transcription factor	Os.4901.1.S1_at	LOC_Os01g49160	4.375	0.002
NAC transcription factor	Os.35020.1.S1_at	LOC_Os11g03300	5.104	0.000
NAC transcription factor	Os.17286.1.S1_at	LOC_Os03g21060	4.050	0.009
Other genes				
Cytochrome P450 CY89A2	Os.46872.1.S1_at	LOC_Os10g37160	41.434	0.001
Cytochrome P450 CY94A1	Os.36161.1.S1_at	LOC_Os01g58960	19.997	0.000
Cytochrome P450 CY72A1	Os.773.1.S1_s_at	LOC_Os01g43750	19.137	0.000
Cytochrome P450 CY72A1	Os.770.1.S1_at	LOC_Os01g43720	17.318	0.000
Cytochrome P450 CY76C2	Os.19393.1.S1_at	LOC_Os02g36110	12.114	0.005
Cytochrome P450 CY94A1	Os.54318.1.S1_at	LOC_Os11g04290	10.818	0.000
Unknown function				
Hypothetical protein	OsAffx.30150.1.S1_at	LOC_Os04g45510	89.224	0.000
Integral membrane protein	Os.55511.1.S1_at	LOC_Os04g45520	69.787	0.000
Expressed protein	Os.55906.1.S1_at	LOC_Os02g37380	54.848	0.000

Table 4. Down-regulation of Fe-responsive genes in the iron-excess susceptible cultivar BR-IRGA 409.

Functional categories	Probe set ID	TIGR gene Locus	Fold change	*P* value
Metal transport/Homeostasis				
OsYS7 (Fe^{3+}-phytosiderophore transporter)	Os.10018.1.S1_at	LOC_Os02g43410	109.301	0.000
OsIRT1 (Fe^{2+} transporter)	Os.19632.1.S1_at	LOC_Os03g46470	38.570	0.000
OsNRAMP1 (putative metal transporter)	Os.409.1.S1_at	LOC_Os07g15460	13.208	0.000
OsYS6 (Fe^{3+}-phytosiderophore transporter)	OsAffx.2947.1.S1_at	LOC_Os02g43370	12.135	0.000
OsYS14 (Fe^{3+}-phytosiderophore transporter)	Os.10024.1.S1_at	LOC_Os04g45900	3.671	0.000
OsNAS1(nicotianamine synthase)	Os.1478.1.S1_at	LOC_Os03g19436	102.149	0.000
OsNAS2 (nicotianamine synthase)	Os.9311.1.S1_at	LOC_Os03g19420	63.421	0.000
OsNAAT1 (nicotianamine aminotransferase)	Os.7989.1.S1_at	LOC_Os02g20360	27.718	0.000
Cell Wall Lignification				
Dirigent-like protein	OsAffx.18732.1.S1_at	LOC_Os11g07740	3.268	0.000
Dirigent-like protein	OsAffx.30868.1.S1_at	LOC_Os11g07770	8.848	0.000
Dirigent-like protein	OsAffx.30449.1.S1_at	LOC_Os10g18870	57.646	0.000
Transport				
POT family protein	Os.32686.1.S1_at	LOC_Os01g65110	11.204	0.001
Tetracycline transporter protein	Os.54195.1.S1_at	LOC_Os11g05390	10.996	0.000
Sulfate transporter	Os.5566.1.S1_s_at	LOC_Os03g09980	8.079	0.000
Sulfate transporter	Os.19822.1.S1_at	LOC_Os03g09970	3.259	0.008
Major facilitator superfamily antiporter	Os.18707.1.S1_at	LOC_Os11g04020	6.201	0.000
Abiotic/Biotic stress related				
Pathogenesis-related protein 10	Os.47802.1.A1_at	LOC_Os12g36840	14.282	0.000
OsPrx95	Os.20290.1.S1_at	LOC_Os07g01410	4.214	0.001
OsPrx78	Os.27789.1.A1_at	LOC_Os06g20150	4.357	0.006
OsPrx41	Os.11561.2.S1_a_at	LOC_Os03g22010	9.312	0.000
OsPrx20	Os.15894.1.A1_a_at	LOC_Os01g73170	6.228	0.0013
OsPrx12	Os.35123.1.S1_at	LOC_Os01g22230	5.627	0.001
OsPrx107	Os.10029.1.S1_at	LOC_Os07g44550	8.423	0.001
OsPrx95	Os.20290.1.S1_at	LOC_Os07g01410	4.214	0.001
Jasmonate induced protein	Os.8510.1.S1_at	LOC_Os10g18760	8.988	0.001
Unknown function				
Expressed protein	Os.12629.1.S1_at	LOC_Os01g45914	30.139	0.000
Unknown protein	Os.26063.1.S1_at	LOC_Os11g15624	12.335	0.000
Expressed protein	Os.18485.1.S1_s_at	LOC_Os03g52680	9.066	0.000
Expressed protein	OsAffx.27066.1.S1_at	LOC_Os05g28770	7.039	0.001
Expressed protein	Os.12788.1.S1_at	LOC_Os01g11240	5.823	0.000

Table 5. Up-regulation Expression profile of Fe-responsive genes in the iron-excess tolerant cultivar EPAGRI 108.

Functional categories	Probe set ID	TIGR gene Locus	Fold change	*P* value
Cell Wall Lignification				
Dirigent-like protein	Os.46700.1.S1_at	LOC_Os10g18820	33.884	0.017
Dirigent-like protein	OsAffx.30449.1.S1_at	LOC_Os10g18870	25.752	0.003
Dirigent-like protein	OsAffx.30868.1.S1_at	LOC_Os11g07770	7.397	0.009
Laccase	Os.51049.1.S1_at	LOC_Os05g38420	3.376	0.007
Copper methylamine oxidase	Os.9293.1.S1_at	LOC_Os04g40040	4.478	0.001
Lignin biosynthesis				
Caffeoyl-CoA O-methyltransferase	Os.738.1.S1_at	LOC_Os08g38910	3.028	0.019
O-methyltransferase ZR4	OsAffx.30947.1.S1_at	LOC_Os11g12760	3.621	0.035
Cell Wall Biosynthesis				
Glycine-rich protein	Os.46476.1.S1_at	LOC_Os10g31670	3.981	0.002
Glycine-rich protein	Os.46670.1.S1_at	LOC_Os10g31560	3.473	0.004
OsXTH6	Os.23174.1.S1_at	LOC_Os08g14200	3.630	0.36
OsXTH10	Os.27205.1.S1_at	LOC_Os06g48180	5.211	0.007
OsXTH18	Os.22839.1.S2_at	LOC_Os06g48200	3.858	0.047
Abiotic/Biotic stress related				
OsPrx78	Os.27789.1.A1_at	LOC_Os06g20150	4.564	0.003
OsPrx20	Os.15894.1.A2_at	LOC_Os01g73170	8.349	0.001
OsPrx107	Os.10029.1.S1_at	LOC_Os07g44550	3.068	0.004
OsPrx41	Os.11561.2.S1_at	LOC_Os03g22010	5.792	0.005
Jasmonate-induced protein	Os.8510.1.S1_at	LOC_Os10g18760	11.241	0.004
Unknown function				
Unknown protein	Os.14411.1.S1_at	LOC_Os06g16640	13.423	0.033
Expressed protein	Os.19632.1.S1_at	LOC_Os03g46470	12.099	0.003
Expressed protein	OsAffx.12383.1.S1_at	LOC_Os02g37260	10.862	0.001
Expressed protein	OsAffx.27066.1.S1_at	LOC_Os05g28770	6.003	0.001

Table 6. Down-regulation of Fe-responsive genes in the iron-excess tolerant cultivar EPAGRI 108.

Functional categories	Probe set ID	TIGR gene Locus	Fold change	*P* value
Abiotic/Biotic stress related				
OsAsr3 (ABA, stress, ripening)	Os.27963.1.A1_at	LOC_Os01g72900	10.956	0.012
OsAsr4 (ABA, stress, ripening)	Os.7372.1.S1_at	LOC_Os01g72910	8.137	0.019
Pathogenesis-related protein 10	Os.5051.1.S1_at	LOC_Os12g36830	13.808	0.001
Pathogenesis-related protein 1	Os.19861.S1_at	LOC_Os07g03730	8.860	0.006
OsAOX1b (alternative oxidase)	Os.3406.1.S1_at	LOC_Os04g51160	5.933	0.013
OsPrx57	Os.55918.1.S1_at	LOC_Os04g55740	6.242	0.008
OsPrx46	Os.2961.1.S1_at	LOC_Os03g25340	9.566	0.002
OsPrx135	Os.11549.1.S1_at	LOC_Os12g02080	6.743	0.019
OsPrx131	Os.55595.1.s1_at	LOC_Os11g02100	8.080	0.005
OsPrx111	Os.22086.1.S1_at	LOC_Os07g48020	4.341	0.011
OsPrx110	Os.2237.1.S1_at	LOC_Os07g48010	4.085	0.004
Senescence-associated protein	Os.56016.1.s1_at	LOC_Os03g63620	12.402	0.001
Xylem cysteine proteinase	Os.28964.1.S1_at	LOC_Os01g42790	3.785	0.006
Xylanase inhibitor protein	Os.4867.1.S1_at	LOC_Os08g40680	3.948	0.000
Xylanase inhibitor protein	Os.32890.1.S1-at	LOC_Os11g47600	7.030	0.002
Xylanase inhibitor protein	Os.4681.1.S1_at	LOC_Os08g40690	5.741	0.002
Xylanase inhibitor protein	Os.51920.1.S1_at	LOC_Os11g47590	3.100	0.009
Xylanase inhibitor protein	Os.9347.1.S1_at	LOC_Os11g47580	10.927	0.000
Nucleotide-binding protein				
OsWRKY9	OsAffx.23292.1.S1_at	LOC_Os01g18600	3.245	0.001
OsWRKY77	Os.30657.1.S1_at	LOC_Os01g40260	5.520	0.001
OsWRKY76	Os.25606.1.S1_at	LOC_Os09g25060	7.360	0.029
OsWRKY62	Os.48082.1.S1_at	LOC_Os09g25070	4.513	0.017
OsWRKY19	Os.55597.S1_at	LOC_Os0549620	4.497	0.011
OsWRKY11	Os.30512.1.S1_at	LOC_Os01g43650	5.338	0.002
NAC transcription factor	Os.35343.1.A1_at	LOC_Os11g03370	3.272	0.002
NAC transcription factor	Os.35020.1.S1_at	LOC_Os11g03300	4.884	0.014
Unknown function				
Expressed protein	Os.55906.1.s1_at	LOC_Os02g37380	37.812	0.005
Expressed protein	Os.Affx.30150.1.S1_at	LOC_Os04g45510	12.245	0.002
Expressed protein	Os.Affx.14888.1.s1_at	LOC_Os05g30500	12.218	0.002
Expressed protein	Os.9900.1.S1_at	LOC_Os06g38764	10.612	0.007
Expressed protein	Os.Affx.28727.1.S1_at	LOC_Os07g33320	6.689	0.006
Expressed protein	Os.57211.1.S1_at	LOC_Os06g13880	6.982	0.007
Hypothetical protein	Os.Affx.14600.1.S1_at	LOC_Os05g08830	15.521	0.004
Hypothetical protein	Os.52208.1.S1_at	LOC_Os11g45990	11.421	0.028
Hypothetical protein	Os.51606.1.S1_at	LOC_Os12g0635400	7.011	0.002
Von Willebrand like protein	Os.5809.1.S1_at	LOC_Os11g46000	30.423	0.036
Other genes				
BI 1- associated receptor kinase 1	Os.Affx.19104.1.S1_at	LOC_Os11g31540	38.461	0.002
Bowman-Birk type trypsin inhibitor	OsAffx.19104.1.S1_at	LOC_Os01g03390	10.214	0.001
Cytochrome P450 CY89A2	Os.46872.1.S1_at	LOC_Os10g37160	56.202	0.007
Cytochrome P450 CY76C2	Os.19393.1.S1_at	LOC_Os02g36110	21.565	0.010
Cytochrome P450 CY99A1	Os.16305.1.S1_at	LOC_Os04g10160	18.699	0.004
Cytochrome P450 CY99A1	Os.53296.1.S1_at	LOC_Os04g09920	17.830	0.005
Cytochrome P450 CY71D7	Os.23518.1.A1_at	LOC_Os02g36190	12.917	0.007
Cytochrome P450 CY79A1	Os.27940.1.S1_at	LOC_Os03g37290	6.582	0.009
Cytochrome P450 CY72A1	Os.773.1.S1_s_at	LOC_Os01g43750	5.597	0.013

Figure legends

Figure 1. Effects of excess iron in leaves (A and B) and roots (C and D) from EPAGRI 108 (A and C) and BR-IRGA 409 (B and D) rice plants exposed for nine days to control (left) and excess (right) iron treatments. Bars represent 0.5 cm. Root (E) and shoot (F) dry weight and total chlorophyll (G) of plants exposed for nine days to control (Ctrl) or iron excess (Fe+). Each value represents the mean of six replicates ± standard error. Distinct letters indicate significant difference between means ($P \leq 0.05$).

Figure 2. Net CO_2 assimilation rate (A) from cultivars BR-IRGA 409 (circles) and EPAGRI 108 (triangles) rice plants, exposed to control (Ctrl, closed symbols) or excess (Fe+, open symbols) iron treatments. Gas exchange measurements were performed after one, two, three, six and nine days of exposure to treatments, using the youngest fully expanded leaf from each plant. Each value represents the mean of six replicates ± standard error.

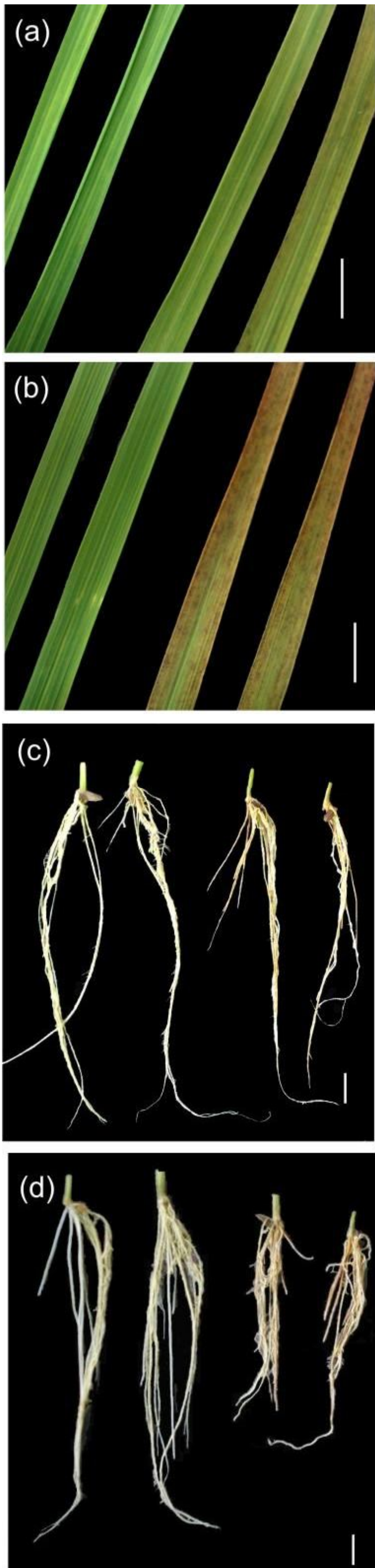
Figure 3. CO_2 response (A and B) and light response (C and D) curves from BR-IRGA 409 (A and C) and EPAGRI 108 (B and D) rice plants after six days of exposure to control (Ctrl, closed circles) or excess (Fe+, open circles) iron treatments. Photosynthetic response curves were obtained for the youngest fully expanded leaf from each plant. Each curve represents the measurements obtained from four independent plants. A = net CO_2 assimilation rate; Ci = estimated substomatal CO_2 partial pressure.

Figure 4. Oxidative damage to lipids and proteins (TBARS and Carbonyl accumulation, respectively) and H_2O_2 accumulation in fully expanded leaves from EPAGRI 108 and BR-IRGA 409 rice plants after nine days of exposure to control (Ctrl) or excess (Fe+) iron treatments. Each value represents the mean of six replicates ± standard deviation. Distinct letters indicate significant difference between means ($P \leq 0.05$).

Figure 5. Activity of antioxidant enzymes (CAT, APX and SOD) in fully expanded leaves from EPAGRI 108 and BR-IRGA 409 rice plants after nine days exposure to control (Ctrl) or excess (Fe+) iron treatments. Each value represents the mean of six replicates ± standard deviation. Distinct letters indicate significant difference between means ($P \leq 0.05$).

Figure 6. Scatter plots of the transformed microarray data from the rice cultivars EPAGRI 108 (tolerant) and BR-IRGA 409 (susceptible to iron excess) exposed to control (Ctrl) or iron excess (Fe+) treatments.

Figure 1



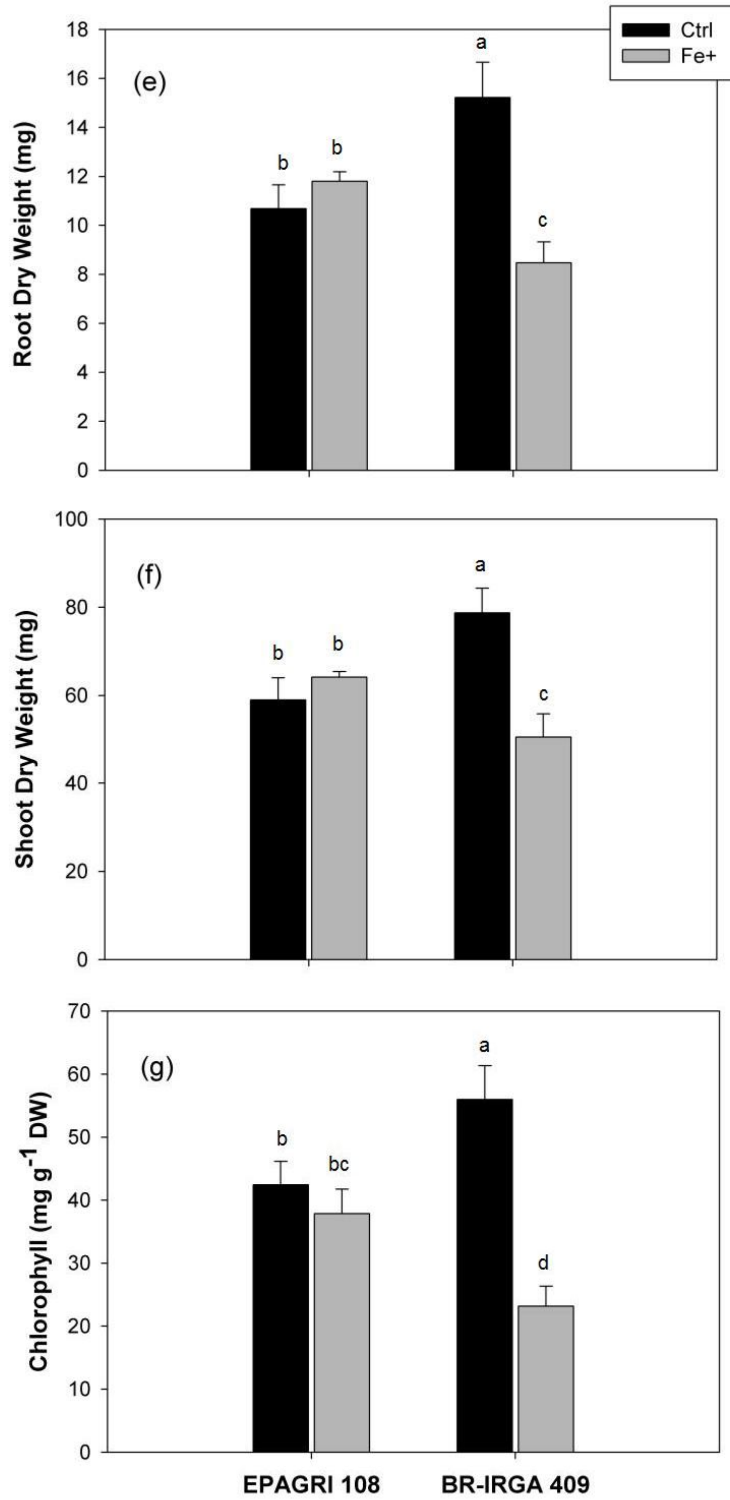


Figure 2

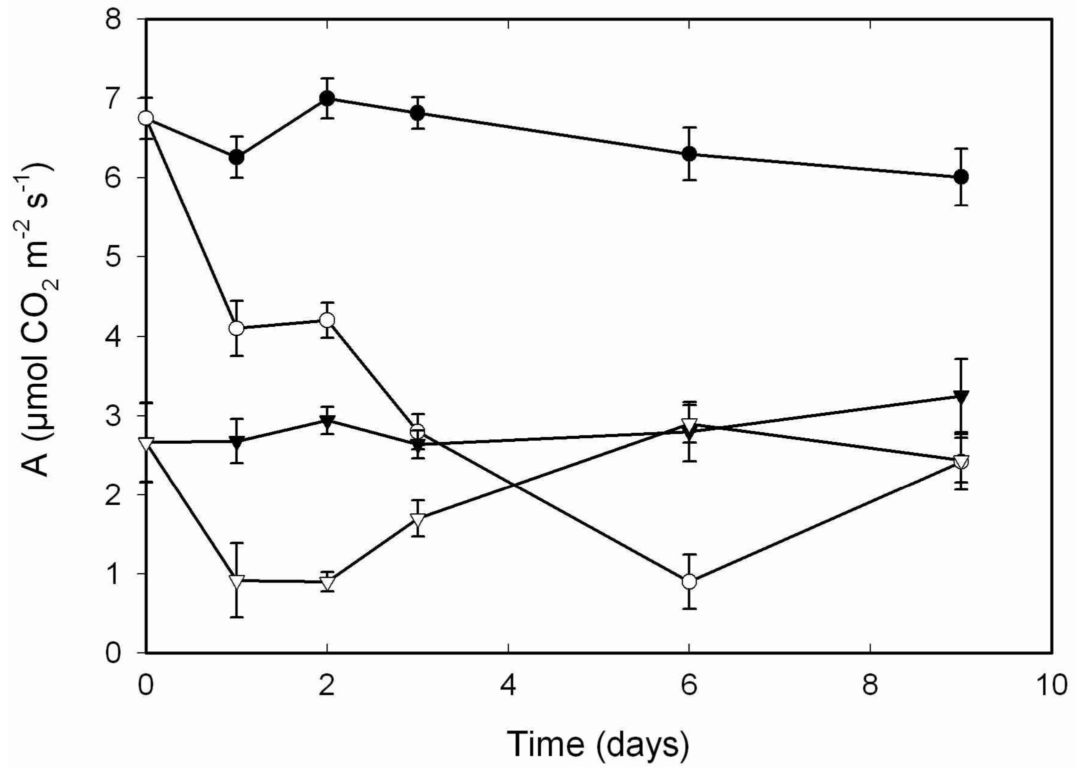


Figure 3

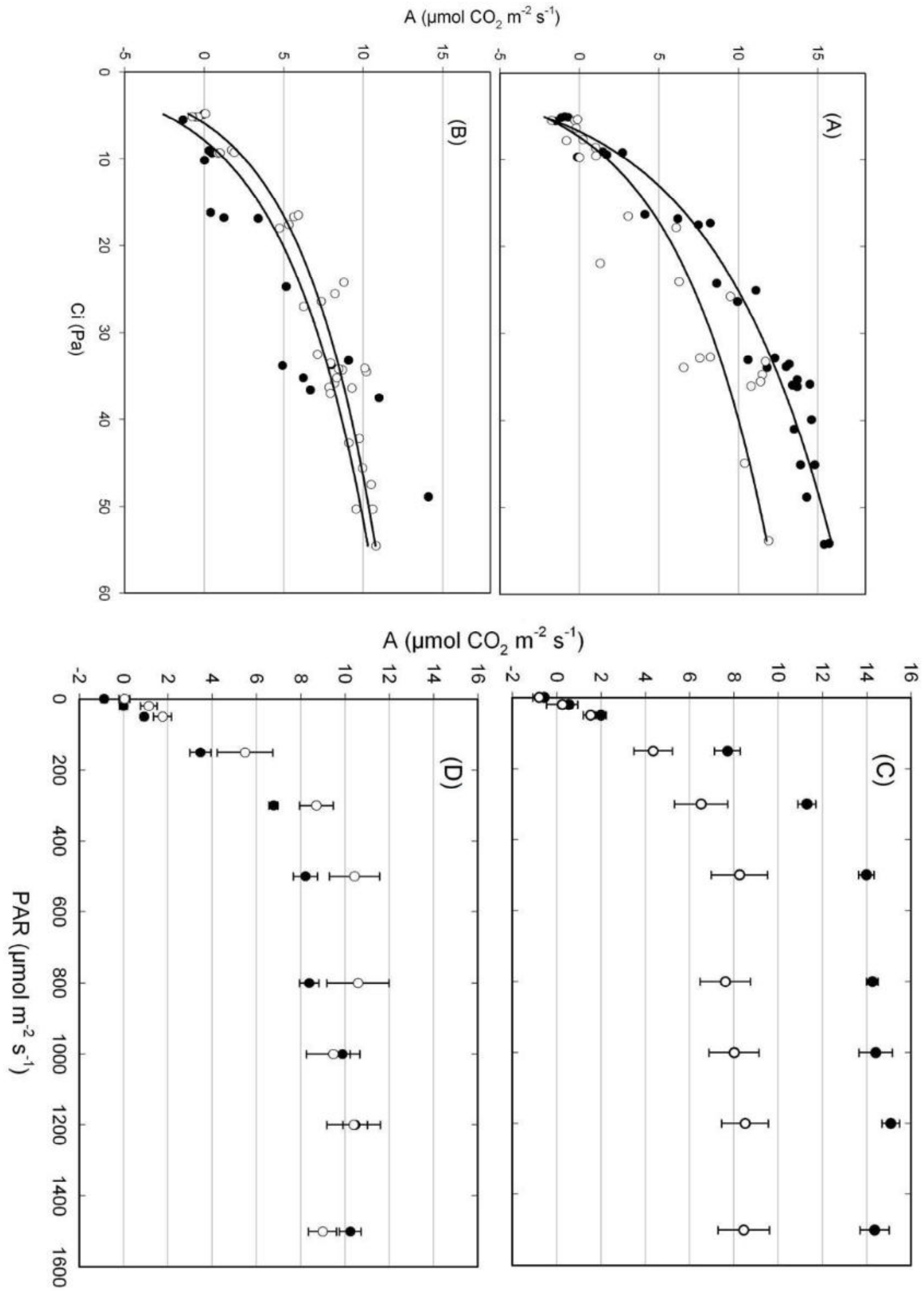


Figure 4

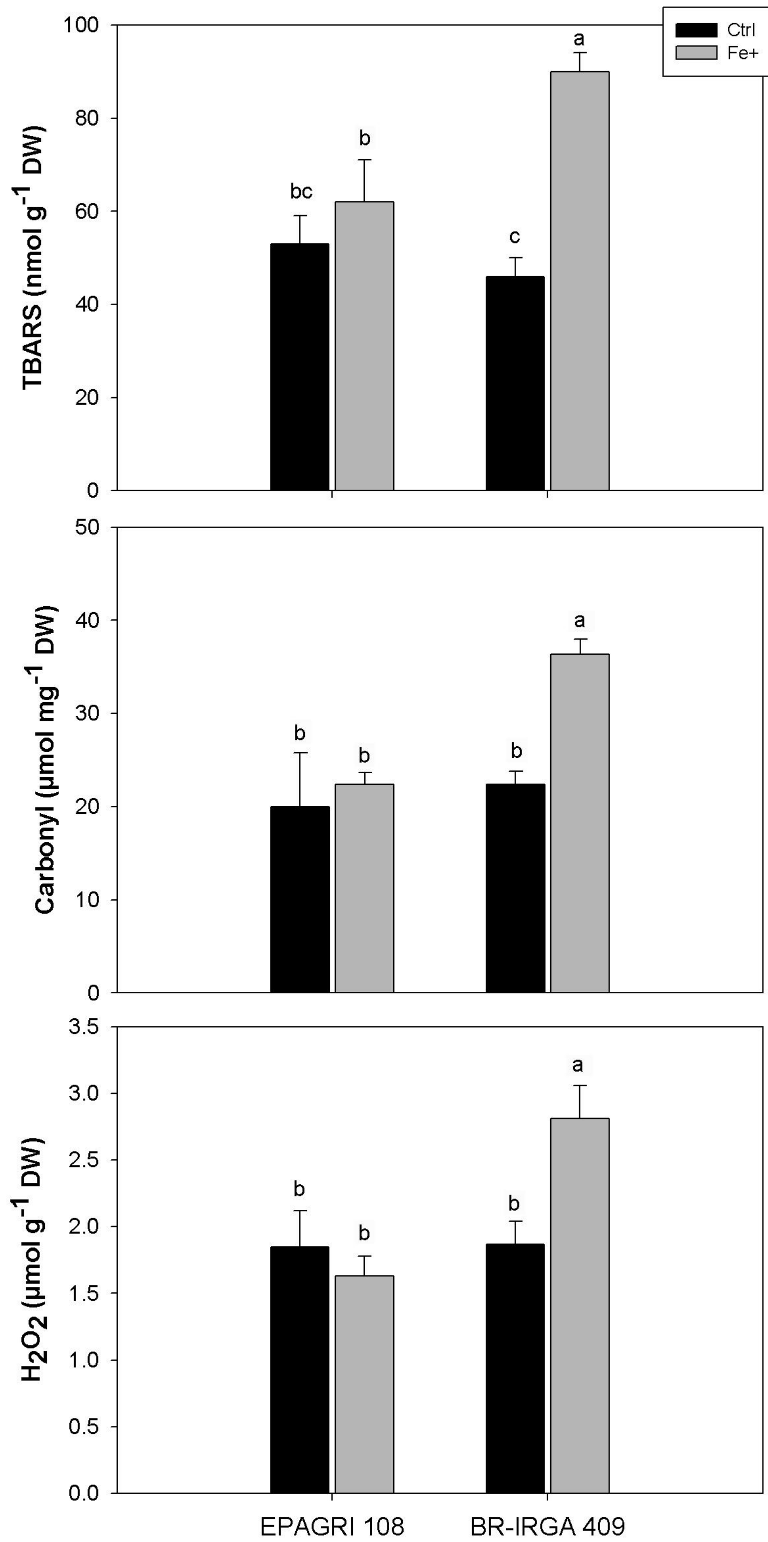


Figure 5

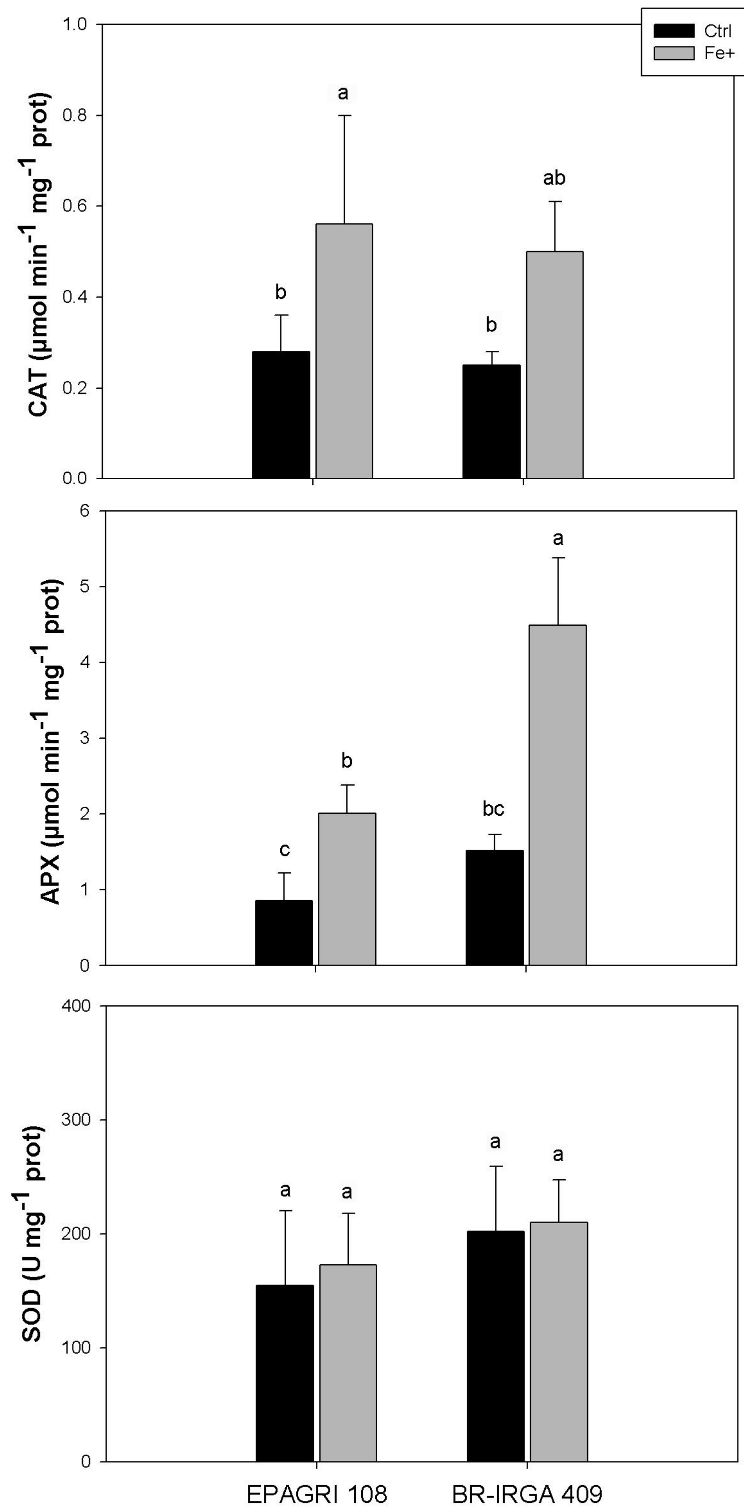
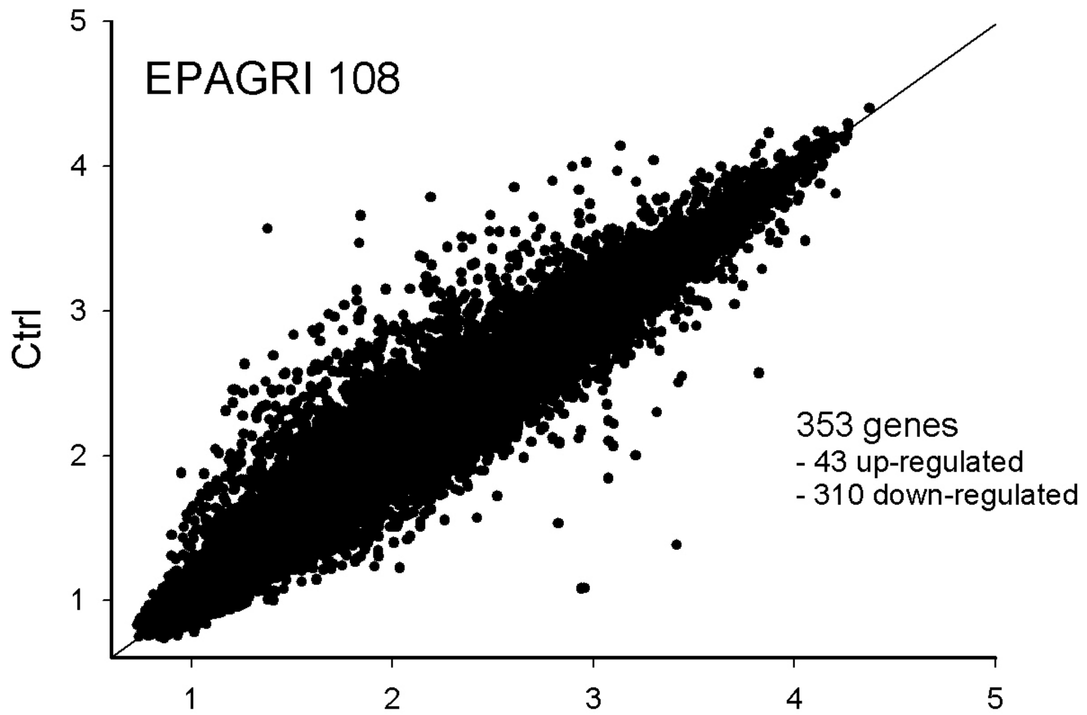
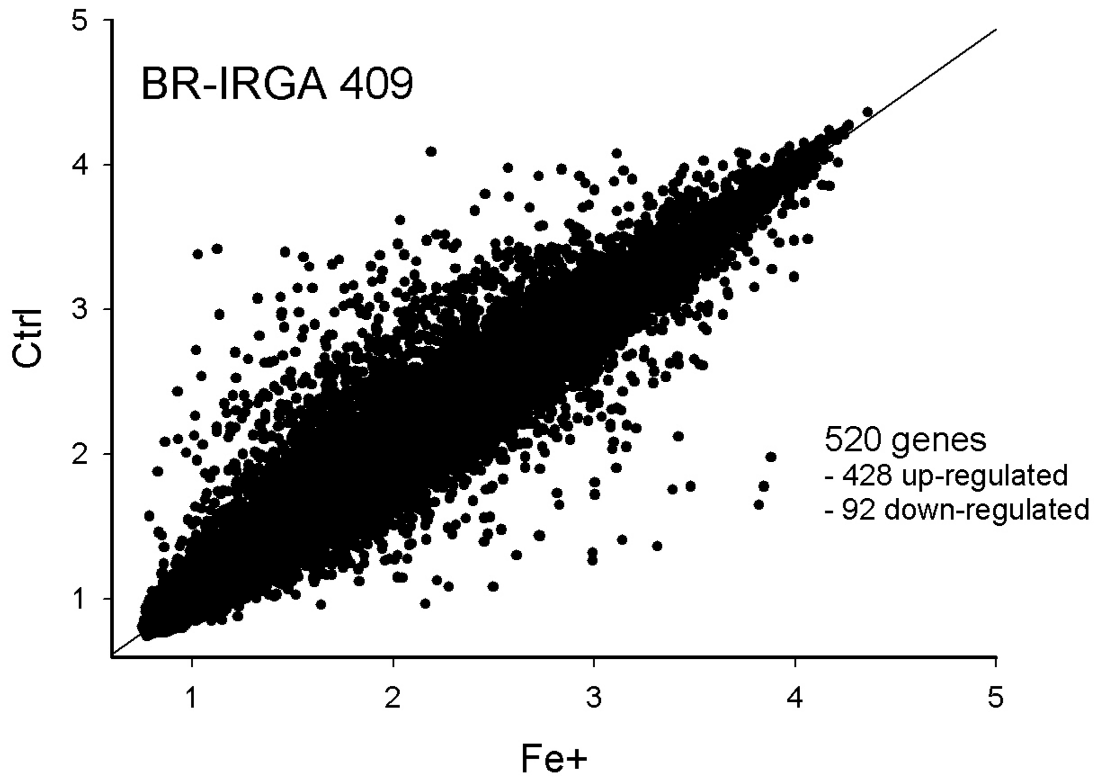


Figure 6





Capítulo 4

"Differential regulation of the two ferritin rice genes (*OsFER1* and *OsFER2*)"

Short Running Title**: Ferritin gene expression in rice**

Differential regulation of the two rice ferritin genes (*OsFER1* and *OsFER2*)

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Abstract

Iron is an essential nutrient to plants. However, when free and in excessive levels inside the cells, iron can catalyze the formation of oxygen free radicals, leading to oxidation of lipids, proteins and nucleic acids. Ferritin, a multimeric spherical protein capable of storing up to 4,500 atoms of iron, acts as an iron buffer in plants. We have previously described two very similar ferritin genes in rice. Using a strategy based in amplicon size difference, we were able to analyze the expression profile of these two genes (*OsFER1* and *OsFER2*). Both genes are expressed, although with different regulation and organ distribution. Exposure to excess iron led to accumulation of ferritin mRNA, remarkably of *OsFER2*. The iron-induced expression was completely abolished by treatment with GSH, indicating that the ferritin mRNA induction observed is dependent of an oxidative step. Exposure to the herbicide Paraquat (an anion superoxide generator) and to SNP (a nitric oxide precursor) also resulted in increased expression of *OsFER2*. Ferritin expression was induced in leaves of rice plants exposed to higher levels of iron, but not in roots. *OsFER2* mRNA accumulation in rice flag leaves and panicles at different reproductive stages was also higher than *OsFER1* mRNA accumulation. No ferritin mRNA was detected in rice seeds. However, 48 and 96 hours of imbibition under light led to ferritin expression (mostly *OsFER2)*. This ferritin mRNA induction was completely abolished when seeds were kept in the dark, suggesting a light-regulated induction. Ferritin mRNA accumulation was seen in the dark only when seeds were germinated in the presence of externally supplied iron. This is the first report on gene expression of the two rice ferritin genes. It is suggested that their primary role is related to defense against iron-mediated oxidative stress.

Introduction

Iron is an essential nutrient for virtually all organisms. In plants, it is involved in essential biological processes, such as photosynthesis, respiration and nitrogen assimilation. Iron deficiency can induce chlorosis and decreases photosynthetic activity, leading to lower plant productivity (Hansen *et al.*, 2006), also leading to leaf and root senescence (Sperotto *et al.*, 2007; Sperotto *et al.*, 2008). Due to its redox properties, excessive amounts of free iron can catalyze the generation of reactive oxygen species (ROS) through the Fenton reaction (Becana *et al.*, 1998). The iron-mediated oxidative stress can cause damage to several biological macromolecules, such as lipids, proteins and nucleic acids (Halliwell & Gutteridge, 1984). One of the major nutritional disorders in irrigated and in lowland rice is iron toxicity, caused by high levels of ferrous iron, solubilized from iron oxides in flooded soils (Ponnamperuma, 1972). Thus, iron homeostasis in plants must be tightly regulated. The iron storage protein ferritin acts as an iron buffer inside the cell by storing this ion in a soluble, non-toxic and bioavailable form. Ferritin is a spherical protein, organized in a 24-subunit shell, containing up to 4,500 atoms of iron in its central cavity (Harrison & Arosio, 1996).

In plants, ferritin is mainly localized in plastids, being also found in mitochondria (Zancani *et al.*, 2004). Plants tend to have small ferritin gene families, with their members being differentially regulated. *Arabidopsis* has four ferritin genes (Petit *et al.*, 2001a), while maize and tobacco have two (Fobis-Loisy *et al.*, 1995; Jiang, 2005).

Ferritin post-transcriptional regulation is well known in animals, where conserved regulatory promoter sequences named IREs (Iron Regulatory Elements) are responsible for the translational de-repression of ferritin mRNA in response to iron overload (Theil, 1998).

Plant ferritin genes do not contain IREs, and regulation is considered to happen mostly at the transcriptional level (Briat *et al.*, 1999). Scarce examples of pos-transcriptional regulation of ferritin genes have been documented in plants (Briat *et al.*, 2006, and references therein).

Plant ferritins are induced under oxidative stress. Iron storage inside ferritin prevents free-radical producing reactions, and ferritin synthesis is regulated by pro-oxidant treatments such as H_2O_2 (Savino *et al.*, 1997), NO donors and scavengers (Murgia *et al.*, 2007; Murgia *et al.*, 2002) and ozone applications (Murgia *et al.*, 2001). The toxic effect of the herbicide Paraquat requires free iron to take place, and plants overexpressing ferritin are more resistant to its toxicity (Deák *et al.*, 1999). Recently, Ravet *et al.* (2008) showed that *Arabidopsis* ferritins are essential to protect cells from iron-derivated oxidative damage and the lack of ferritin leads to reduced growth and strong defects in flower development. Overexpressed ferritin proteins have also been considered as potential iron sources in grain biofortification studies (Zhu *et al.*, 2007).

Our group identified two ferritin genes in the rice genome, which share 96% identity (Gross *et al.*, 2003). The differences between the two complete rice ferritin cDNAs are three deletions in *OsFER1* (two located in the 5'-untranslated region and the other immediately downstream from the start codon), as well as 15 single nucleotide changes within the coding region. Both genes are located in a recently duplicated region present in chromosomes 11 and 12, what could explain the high similarity between the two rice ferritin genes (Gross *et al.*, 2003). The African rice *Oryza glaberrima* is the only related species in which ferritin gene regulation by iron has been documented (Majerus *et al.*, 2007). This is the first report of ferritin gene regulation in *Oryza sativa* in which expression of each one of the two genes was independently evaluated. A strategy based on

differences in amplicon sizes allowed us to analyze separately the expression profile of the very similar ferritin genes in rice plants, investigating whether both rice ferritin genes are expressed and how different treatments shown to influence ferritin expression in other plant species regulate the two rice ferritin genes.

Materials and Methods

Treatments and plant material

Seeds from *Oryza sativa* ssp. *japonica* (cultivar Nipponbare) were surface sterilized and germinated on moistened Petri dishes for 48 hours. Seedlings were kept in water for additional 48 hours and then transferred to different treatments: control (H_2O), Cu (1mM $CuSO_4$), SNP (1.5 mM), Paraquat (30 µM), H_2O_2 (5 mM), Fe (5 mM $FeSO_4$), Fe (5 mM $FeSO_4$) + GSH (5 mM), GSH (5 mM), GSSG (2.5 mM) and ABA (100 µM). For the combined treatment (Fe + GSH) seedlings were pre-incubated with GSH (5 mM) for two hours and then subjected to Fe (5 mM) plus GSH (5 mM).

Seedlings were harvested after 6, 12, 24 and 48 hours of exposure to the respective treatments and stored at -80°C. For the seed germination experiment, surface sterilized seeds were germinated on Petri dishes and samples were harvested after 0, 24, 48 and 96 hours of imbibition in MES (3 mM, pH 6.0) or Fe (MES 3 mM, pH 6.0 + 100 µM Fe^{3+}-citrate) and were kept in light or dark conditions.

For the analysis of ferritin regulation by iron, plants were grown as described in Silveira *et al.* (2007) and subjected to iron excess (9 mM Fe^{2+}, supplied as $FeSO_4$), deficiency (with no iron source, plus 0.15 mM ferrozine – a free ferrous iron chelator) and control treatment (0.12 mM Fe^{2+}). Leaf and root samples were harvested after 0, 24 and 96 hours of exposure

to the treatments. Flag leaves and panicles were obtained from rice plants (cultivar Nipponbare) cultivated in the field (at the Instituto Rio Grandense do Arroz in Cachoeirinha, Brazil - 29º 56’ 51.91’’S, 51º 06’ 46.36’’W). The distinct reproductive stages were recognized according to Counce *et al.* (2000) and samples were harvested at R3 (pre-anthesis), R4 (anthesis), R5 (grain filling) and R7 (grain maturity). Panicles at the R4 stage were dissected and the palea, lemma, anther and carpel were harvested and immediately frozen.

Rice fully expanded leaves were used for dark induction of senescence. Detached leaves (0.5 cm^2) were kept in the dark while subjected for 0, 1, 3 and 5 days to the following treatments: control (MES 3 mM, pH 5.8), BAP (BAP 50 µM + MES 3 mM, pH 5.8) and ABA (ABA 50 µM + MES 3 mM, pH 5.8). Plant material were collected and immediately frozen (-80°C). As a senescence marker, we analyzed the expression of the *OsSgr* gene (*staygreen*), a senescence-associated gene encoding a chloroplast protein (Park *et al.*, 2007; primers 5’-CTACCAAACCGAGCCAAAT-3’, 5’-ACCAAAACGACTCTTGACAGC-3’).

RNA isolation and cDNA synthesis

Total RNA was isolated from seedlings, leaves and roots using Trizol (Invitrogen) or from flag leaves, panicles, reproductive organs and germinating seeds using the Concert Plant RNA Purification Kit (Invitrogen), according to manufacturer’s instructions. RNA integrity was confirmed in 1.6 % agarose gels stained with ethidium bromide and RNA was quantified with the Quant-iT RNA Assay Kit and the Qubit Fluorometer (Invitrogen). One microgram of total RNA was treated with DNAse (Invitrogen) and reverse transcribed using M-MLV (Invitrogen).

RT-PCR analysis

Semi-quantitative RT-PCR was performed using standard conditions (Sperotto *et al.*, 2008) and PCR products analyzed in the linear phase of amplification (using 33 PCR cycles for ferritin and 30 PCR cycles for ubiquitin and actin). Amplification of the two ferritin mRNA isoforms was performed using a strategy based on the difference of amplicon sizes (Figure 1) using the following primers: forward (5'–TCACTCTTCACCCGCCGCG–3') and reverse (5'–TCGACGAACTTTTGCCTAGC–3'). Amplification of *OsFER2* generates a 338 bp amplicon, and of *OsFER1* a 309 bp amplicon, shown as cDNA amplification products in *Fig*. 1. PCR products were resolved in 1.6% agarose gels stained with ethidium bromide and the constitutive products of ubiquitin (Miki *et al.,* 2005) or actin (Yokoyama *et al.*, 2004) were used as controls. Actin amplification was used as control only for the analysis of dissected panicle organs, since the abundance of ubiquitin transcripts varied between those samples. As a positive control to the iron deficiency treatment, we analyzed the expression of the genes *OsIRO2* (Ogo *et al.*, 2006) 5'-CCACAGGAAGCTCAGCCACA-3'; 5'-CAGATTCTCCACCTGCTTCTGC-3') and *OsYSL15* (Koike *et al.* 2004), 5'-GGATTGCAGAAATAAACAGTGATG-3; 5'-TGCCAAACTAAACAATTCTCAA-3') in leaves and in roots, respectively.

Results

Ferritin expression in seedlings

To study the expression profile of the rice ferritin genes, we subjected rice seedlings to distinct treatments and analyzed the accumulation of ferritin mRNA. The abundance of ferritin transcripts (*OsFER1* and *OsFER2*) was distinctly altered by the treatments (Figure

2). Exposure to Cu and Paraquat, two treatments that induce oxidative stress, resulted in increased expression of *OsFER2* in rice seedlings (Figure 2). Expression of the rice ferritin genes was not affected by H_2O_2 treatment (Figure 2).

Expression of both *OsFER1* and *OsFER2* increased upon exposure to SNP (a nitric oxide donor), although *OsFER2* accumulation was clearly higher (Figure 2). The involvement of nitric oxide in the induction of the ferritin genes was also demonstrated in *Arabidopsis* by Murgia *et al.* (2002), with nitric oxide functioning as a signal molecule (Arnaud *et al.*, 2006), acting downstream from iron overload.

Treatment with 5 mM of iron resulted in higher *OsFER2* expression in all time points tested, and despite the accumulation of *OsFER1* after 24 hours, iron treatment induced preferably the accumulation of *OsFER2* transcripts (Figure 2). Iron is a strong inducer of at least one ferritin gene in each one of several plants tested, such as maize, *Arabidopsis*, soybean, *Oryza glaberrima* and tobacco (Fobis-Loisy *et al.*, 1995; Gaymard *et al.*, 1996; Lescure *et al.*, 1991; Majerus *et al.*, 2007; Jiang, 2005). We found a similar pattern in rice, with one isoform being preferably induced.

To further characterize the iron-induction of *OsFER2*, seedlings were pre-treated with GSH (an antioxidant molecule) prior to the iron treatment. In these samples, the iron-induction of *OsFER2* expression was completely abolished (Figure 2), suggesting a dependence of an oxidative step for *OsFER2* induction. Exposure to GSH alone or to its oxidized form, GSSG, had no effect on ferritin mRNA accumulation. The ratio between reduced and oxidized glutathione seems to be important in triggering plant responses to different stresses (Tausz *et al.*, 2004). Our results suggest that the oxidative status of glutathione does not operate in the oxidative induction of *OsFER2* expression.

The stress-hormone ABA did not affect the *OsFER1* and *OsFER2* mRNA abundance in seedlings (Figure 2).

Iron regulation in leaves and roots

To analyze the effect of iron on ferritin mRNA abundance in more detail, we subjected plants to distinct iron concentrations (normal, excess and deficiency levels). Iron excess induced expression of *OsFER1* and *OsFER2* in leaves after 24 hours and at higher level after 96 hours (Figure 3A). However, such strong increase in ferritin mRNA accumulation could not be seen in roots (Figure 3B).

Iron deficiency did not result in clear reduction of ferritin expression in relation to the control treatment, neither in leaves nor in roots (Figure 3A and 3B). The accumulation of *OsIRO2* and *OsYSL15*, two genes known to be induced by iron deficiency in rice shoots and roots, respectively (Ogo *et al.*, 2006; Koike *et al.*, 2004) increased with the exposure to the iron deficiency treatment (Figure 3), confirming the effectiveness of the treatment used in our experiments.

Ferritin expression in reproductive organs

Ferritin transcript accumulation was also examined in rice reproductive organs (flag leaves and panicles) in different developmental stages. Only *OsFER2* expression could be detected during the analyzed developmental stages of the rice flag leaf (Figure 4A). Despite small accumulation of *OsFER1* seen in panicles (Figure 4B), *OsFER2* was also more abundant in these organs. To detail the accumulation of ferritin transcripts observed in panicles, we dissected panicles at the R4 stage (anthesis) in palea, lemma, anther and carpel. Ferritin mRNA accumulation (both *OsFER1* and *OsFER2*) was detected in all

organs tested, but at lower level in anthers (Figure 4C). In contrast to the very low accumulation of *OsFER1* in complete panicles, both ferritin transcripts were found in the specific dissected organs. The distinct pattern of *OsFER1* and *OsFER2* expression between the whole panicle and the dissected organs in the same reproductive stage may be a result of differential gene expression in panicle structures not included in the specific dissected organ analysis, such as the rachis.

Regulation of ferritin genes during seed germination

To investigate the role of ferritin through seed germination in rice, seedlings were imbibed in MES (pH 6.0) or Fe (MES pH 6.0 + 100 µM Fe^{3+}-citrate) and the ferritin mRNA accumulation followed through time with plants under light or in the dark. There was no signal of ferritin expression in dry seeds (Figure 5). However, expression of both isoforms was seen after 48 hours and very strong expression of *OsFER2* was detected after 96 hours of imbibition in MES in light conditions (Figure 5A). Interestingly, the ferritin induction could not be seen in the dark, showing a light-dependent regulation of ferritin mRNA accumulation (Figure 5B*)*. When seeds were germinated with Fe, a similar pattern was observed, with visible induction of *OsFER2* after 48, and increased expression in 96 hours (Figure 5C). However, when seeds were kept in the dark, in contrast to seeds germinated with MES, iron induced the expression of *OsFER2*, readily after 24 hours (Figure 5D). These results suggest that the observed ferritin induction is dependent of two distinct signals (light and Fe), but not in an additive way.

Ferritin expression during dark-induced senescence

No clear induction of ferritin expression could be observed in rice detached leaves (Figure 6), in contrast to the previously reported mRNA accumulation of *AtFer1* during dark-induced senescence in *Arabidopsis* (Murgia *et al.*, 2007) and *LlFer2* and *LlFer3* during nodule senescence in *Lupinus lupus* (Strozycki *et al.*, 2007). As expected, *OsSgr* mRNA abundance increased during the dark-induced senescence and BAP (a senescence antagonist) retarded its accumulation, whereas ABA (a known senescence inductor) showed the inverse effect**,** leading to increased accumulation. These results validate our dark-induced senescence model.

Discussion

Based on differences in amplicon sizes, we analyzed the expression pattern of the two rice ferritin genes separately. Both genes are expressed at the mRNA level. The results obtained with rice seedlings indicate that iron can induce accumulation of ferritin transcripts (and more preferably *OsFER2*) through an oxidative step, but not involving H_2O_2. Moreover, this induction seems to be ABA-independent and not directly regulated by the oxidative status of glutathione.

It is possible that accumulation of the rice ferritin transcripts is dependent on oxidative stress signaling and not only on the metal (iron or copper) concentration *per se*. Indeed, ferritin induction by the herbicide Paraquat (an anion superoxide generator), and the observed inhibition of the iron-mediated accumulation of *OsFER2* by GSH treatment in seedlings support this hypothesis. The protective effect of ferritin from oxidative damage induced by a wide range of stresses such as photoinhibition (Murgia *et al.*, 2001),

Paraquat and pathogen attack was studied in detail in plants overexpressing ferritin genes (Deák *et al.*, 1999). Thus, rather than the presence of the metal itself, one leading regulatory signal could be the metal-generated oxidative stress (seen in our experiments as the increase in *OsFER2* mRNA upon Paraquat treatment). The toxic effect of methylviologen (the active molecule of Paraquat) requires free iron, and can be antagonized by iron chelators such as desferrioxamine (Zer *et al.*, 1994), indicating that the protective effect of ferritin could be involved in the capture of free iron and thereby limiting the oxidative effect generated by Paraquat.

Ravet *et al.* (2008) showed that in *Arabidopsis*, in contrast to pea (Marentes & Grusak, 1998), ferritin does not act as a major source of iron in the initial steps of plant development, but is of a great importance against oxidative stress induced by free iron. It would be tempting to say that in rice, as in *Arabidopsis*, the primary role of ferritins (and primarily *OsFER2*) is to take part in a defense mechanism against iron-mediated stress rather than acting as an iron-storage protein for the initial plant development. In contrast to its localization in pea, iron is mainly localized in the aleurone layer of the rice grain (Krishnan *et al.*, 2001), as phytate-rich inclusions (Prom-u-thai & Rerkasem, 2001). Thereby, ferritins appear to have no major importance as iron-storage proteins in the rice grain.

To support this hypothesis, we germinated seeds in the presence or absence of Fe and kept the seeds under light or in the dark. When seeds were germinated in the light, a strong induction of *OsFER2* gene expression could be seen, but this induction were completely abolished when seeds were germinated in the dark. This light-induced ferritin expression is possibly linked directly with chloroplast development, and ferritins could be involved in a mechanism used to prevent photo-oxidative damage. Despite its plastid localization,

ferritin does not seem to be necessary for the production of a functional chloroplast or for the proper leaf development in *Arabidopsis* (Ravet *et al.*, 2008). Thereby, the observed ferritin mRNA induction during seed germination could be a mechanism used by rice seedlings to deal with photo-oxidative damage.

Interestingly, a similar ferritin induction pattern was seen in the iron and in the control treatments in seeds germinated under light. However, a rapid induction of *OsFER2* could be seen in seeds supplied with iron and germinated in the dark. Light is known to induce expression of several genes involved in the photosynthetic machinery in seedlings (Jiao *et al.*, 2005), and the photosynthetic metabolism is known to have a huge iron demand. Since plants were germinated in the dark and with iron, the induction observed could be a response to excessive iron accumulation. Under light, the high seedling demand for iron could avoid excessive iron accumulation, and ferritin induction could be a response to the light treatment, independent from the iron addition. Therefore, ferritin gene expression during seed germination may involve two distinct pathways, one driven by light and another by iron, in a non-additive way.

Our results provided evidence of differential control of ferritin expression in different organs and developmental stages. Iron overload resulted in increased mRNA accumulation of both ferritin genes in seedlings and in leaves, with *OsFER2* being preferentially induced. However, the same treatment did not induce ferritin expression in roots. In the related species *Oryza glaberrima*, iron excess also induced the accumulation of ferritin transcripts in leaf tissues but not in roots, although higher levels of iron in roots than in leaves were recorded (Majerus *et al.*, 2007).

Expression of the rice ferritin genes does not appear to be regulated by ABA, neither in seedlings or during dark-induced senescence. The *Arabidopsis AtFer1* gene expression in

response to Fe excess is also ABA-independent, and antagonized by antioxidants. The *AtFer1* promoter region contains a functional IDRS (Iron Dependent Regulatory Sequence), responsible for repression of transcription under low iron supply (Petit *et al.*, 2001b). Interestingly, both rice ferritin genes also contain IDRS sequences in their promoter regions (Gross *et al.*, 2003), what could explain a similar regulation pattern between *AtFer1* and the rice ferritin genes. However, other elements are probably involved in regulation of the rice ferritin genes, since their expression is differentially influenced by several of the tested treatments. Moreover, the basal ferritin expression observed in the control treatment was not repressed under iron deficiency, although expression of genes typically induced by iron deficiency (*OsIRO2* in shoots and *OsYSL15* in roots) increased under the same treatment.

The predicted amino acid sequences from the two mature rice ferritin proteins share 96% identity (Gross *et al.*, 2003). Therefore, it is unlikely that variations in protein structure can be solely responsible for different functions. However, the variation in their temporal and spatial expression pattern, allied to the distinct responses to several treatments, suggests that the two rice ferritin genes may have isoform-specific biological roles. In *Arabidopsis*, besides the high structural conservation found between the four ferritin genes (66.3% identity and higher), their mRNA expression differ in response to various environmental signals and during the course of plant growth and development (Petit *et al.*, 2001a), also suggesting that they could have specific biological roles. The specific roles of each one of the ferritin genes in rice plants remains to be clarified, but we suggest that, as in *Arabidopsis*, the primary role of rice ferritins could be related to the defense machinery against iron-mediated oxidative stress, and not to serve as an essential iron source for the early rice development. The characterization of ferritin mutants and siRNA

plants specifically defective in only one isoform, as well as the subcellular localization of each protein isoform, would provide good insights about the *in vivo* function of the two ferritin genes in rice.

Acknowledgements

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Figure Legends

Figure 1. PCR-based strategy used in the analysis of gene expression of rice ferritin genes, *OsFER1* and *OsFER2*. The strategy is based on the difference in amplicon sizes using the primers: forward (5'–TCACTCTTCACCCGCCGCG–3') and reverse (5'–TCGACGAACTTTTGCCTAGC–3'). (A) Schematic representation of the cDNA structure of *OsFER1* (AK059354) and *OsFER2* (AK102242). (B) cDNA amplification products from *OsFER1* (309 bp amplicon) and of *OsFER2* (338 bp amplicon). PCR products were resolved in 1.6% agarose gels and stained with ethidium bromide. Primers are represented out of scale.

Figure 2. Expression of the rice ferritin genes *OsFER1* and *OsFER2* (A) in seedlings exposed for 6, 12, 24 and 48 hours to distinct treatments (control, Cu, SNP, Paraquat, H_2O_2, Fe, Fe + GSH, GSH, GSSG and ABA). Ubiquitin (*OsUbq)* expression (B) was evaluated as a control. Semi-quantitative RT-PCR was performed using standards conditions and the PCR products resolved in 1.6% agarose gels stained with ethidium bromide.

Figure 3. Ferritin gene expression (*OsFER1* and *OsFER2*) in response to distinct iron concentrations in rice leaves (A) and roots (B). Rice plants were exposed to control (Ctrl, 0.12 mM Fe), iron deficiency (Fe-, no iron added, plus 0.15 mM of ferrozine) or iron excess (Fe+, using 9 mM Fe) treatments. Plants were collected after 0, 24 and 96 hours of exposure to the respective treatments, and total RNA isolated and used for cDNA synthesis. Semi-quantitative RT-PCR were performed using standards conditions and the expression

of *OsIRO2* and *OsYSL15* used as positive controls for iron deficiency in leaves and roots, respectively. Ubiquitin (*OsUbq*) expression was used as control.

Figure 4. Ferritin mRNA abundance in flag leaves (A) and panicles (B) during the developmental stages R3 (pre-anthesis), R4 (anthesis), R5 (grain filling) and R7 (grain maturity). Panicles at the R4 stage were dissected into palea, lemma, anther and carpel, and total RNA used for semi-quantitative RT-PCR (C). Semi-quantitative RT-PCR were performed using standards conditions and the expression of ubiquitin (*OsUbq*) or actin (*OsAct*) were used as control for equal loading.

Figure 5. Ferritin gene expression during rice seed germination. Seeds were germinated on Petri dishes and samples harvested after 0, 24, 48 and 96 hours of imbibition in MES buffer (pH 6.0) (A, B) or Fe (MES 3 mM, pH 6.0 + 100 µM Fe^{3+}-citrate) (C, D). Seeds were kept under light (A, C) or in the dark (B, D). Total RNA was isolated and semi-quantitative RT-PCR performed using standard conditions. The mRNA abundance of ubiquitin (Os*Ubq*) genes was used as control for equal loading.

Figure 6. Semi-quantitative RT-PCR expression profile of the rice ferritin genes (*OsFER1* and *OsFER2*) in detached leaves upon dark-induced senescence. Leaf segments (0.5 cm^2) were subjected to control (MES 3 mM, pH 5.8), BAP (BAP 50 µM + MES 3 mM, pH 5.8) and ABA (ABA 50 µM + MES 3 mM, pH 5.8) and harvested after 0, 1, 3 and 5 days of exposure to the treatments. Total RNA was isolated and RT-PCR performed using standard conditions. The mRNA abundance of the staygreen (*OsSgr*) and the ubiquitin (Os*Ubq*) genes were used as a senescence marker and as control, respectively.

Figure 1

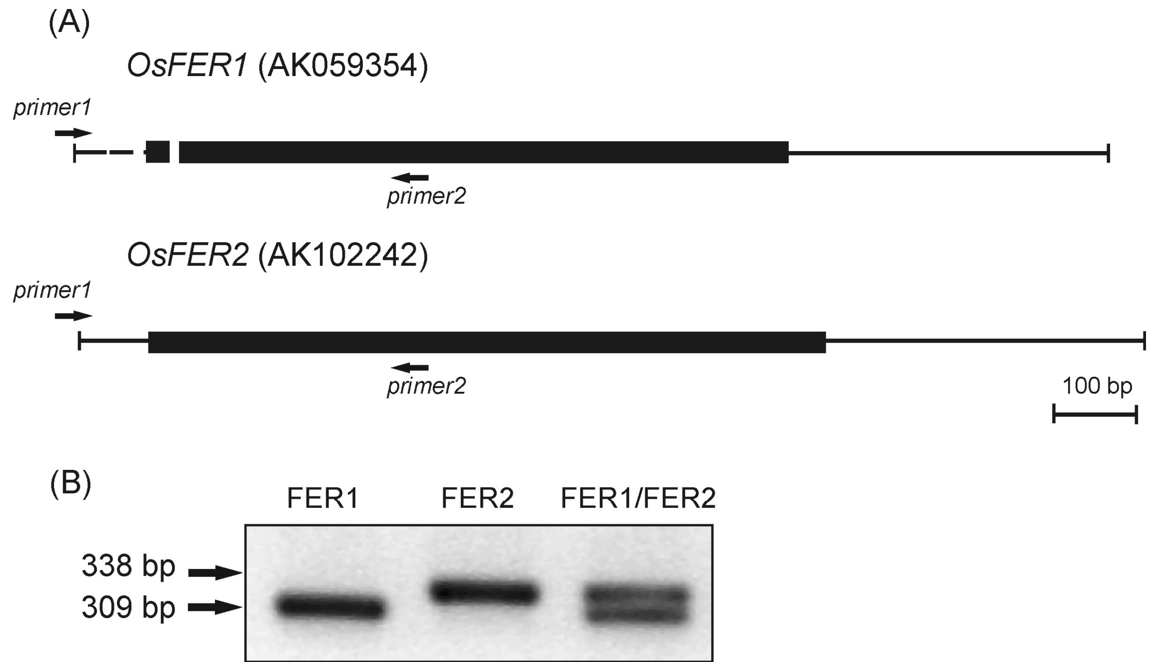


Figure 2

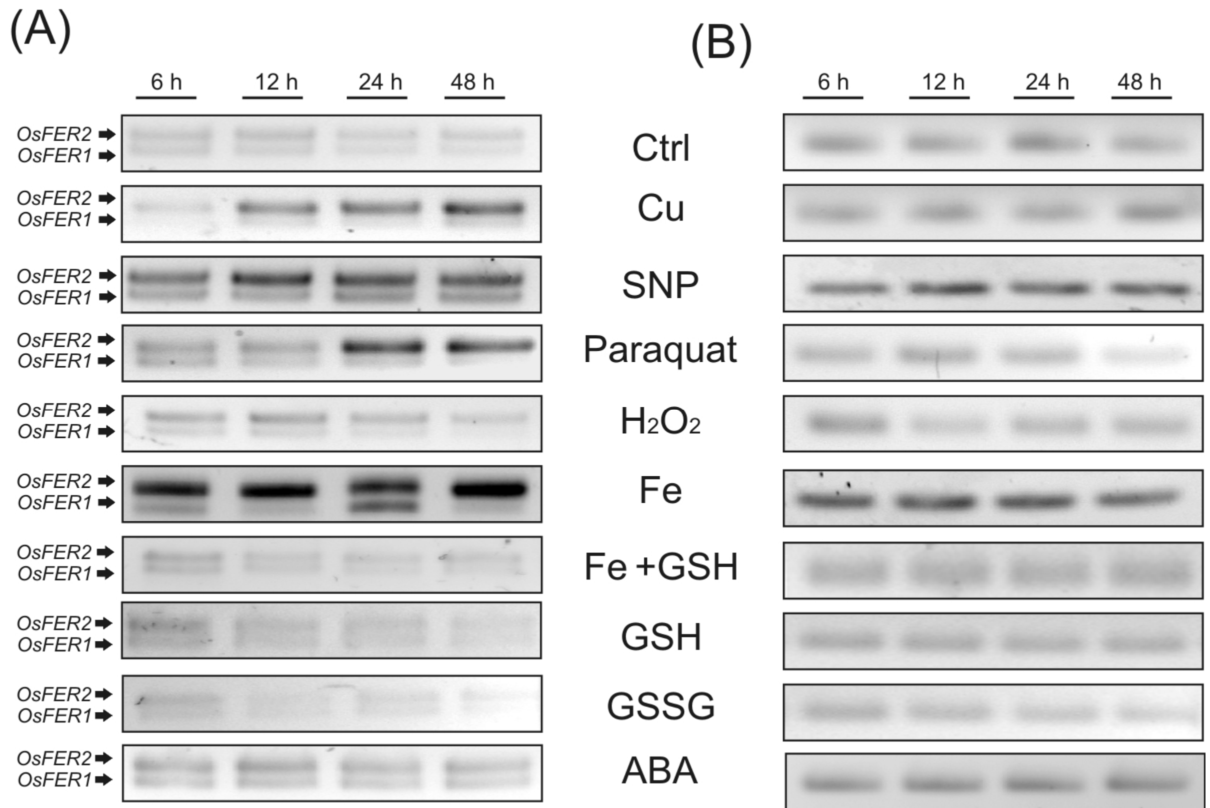


Figure 3

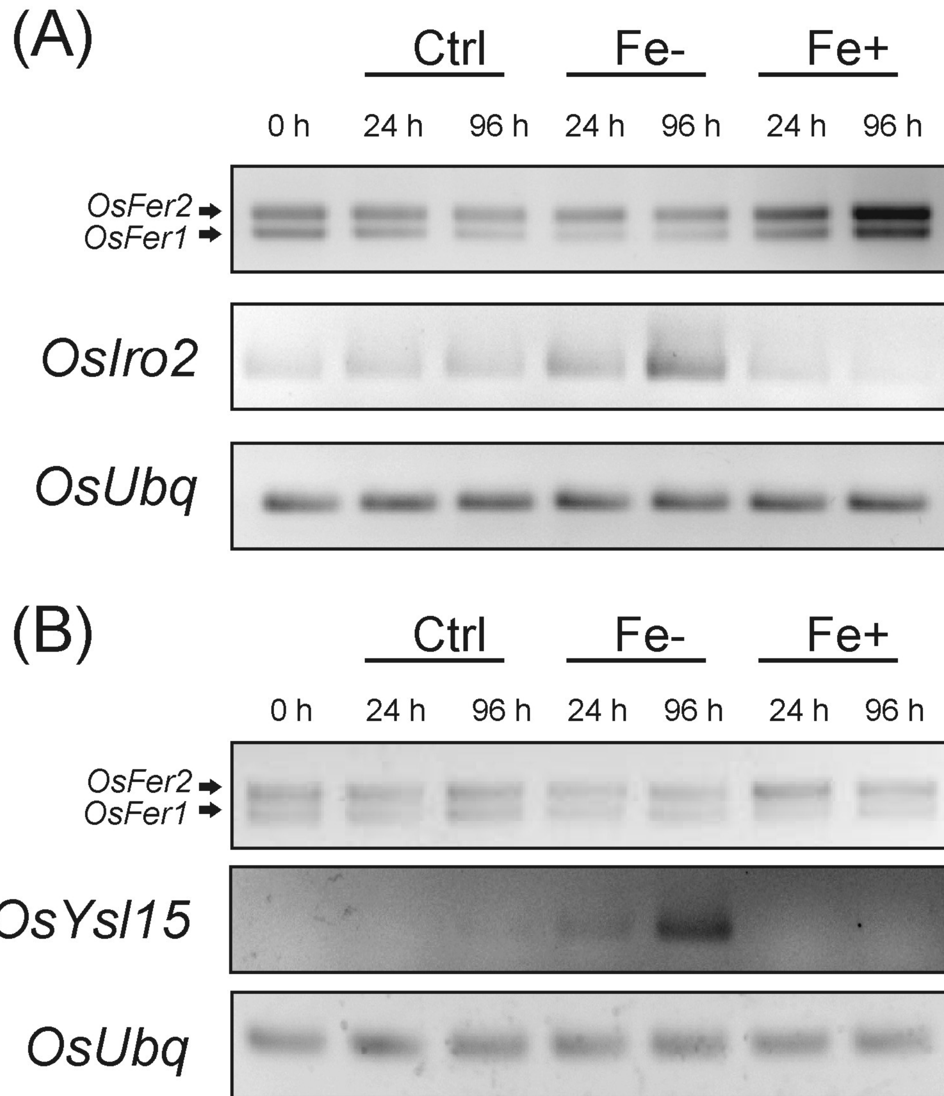


Figure 4

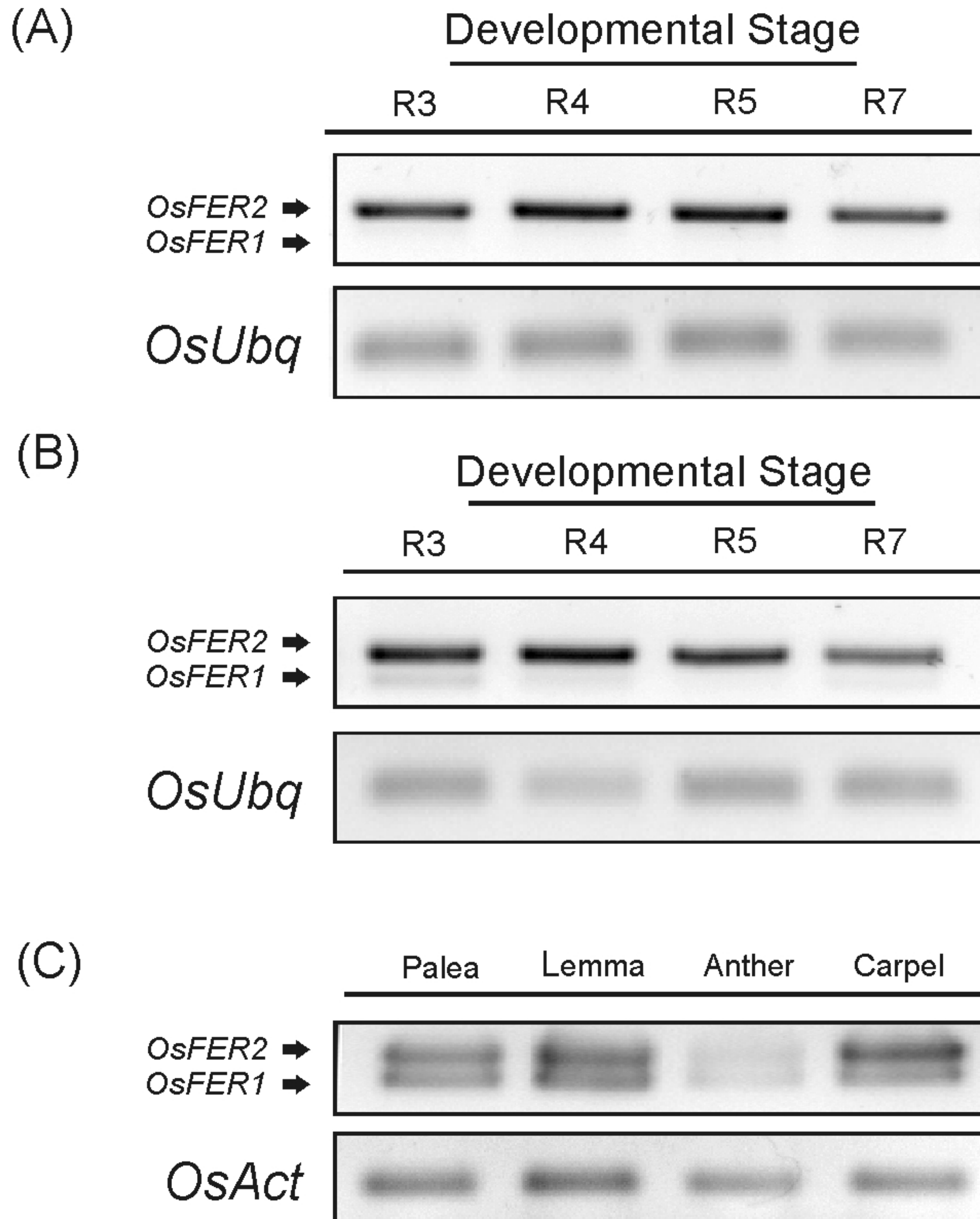


Figure 5

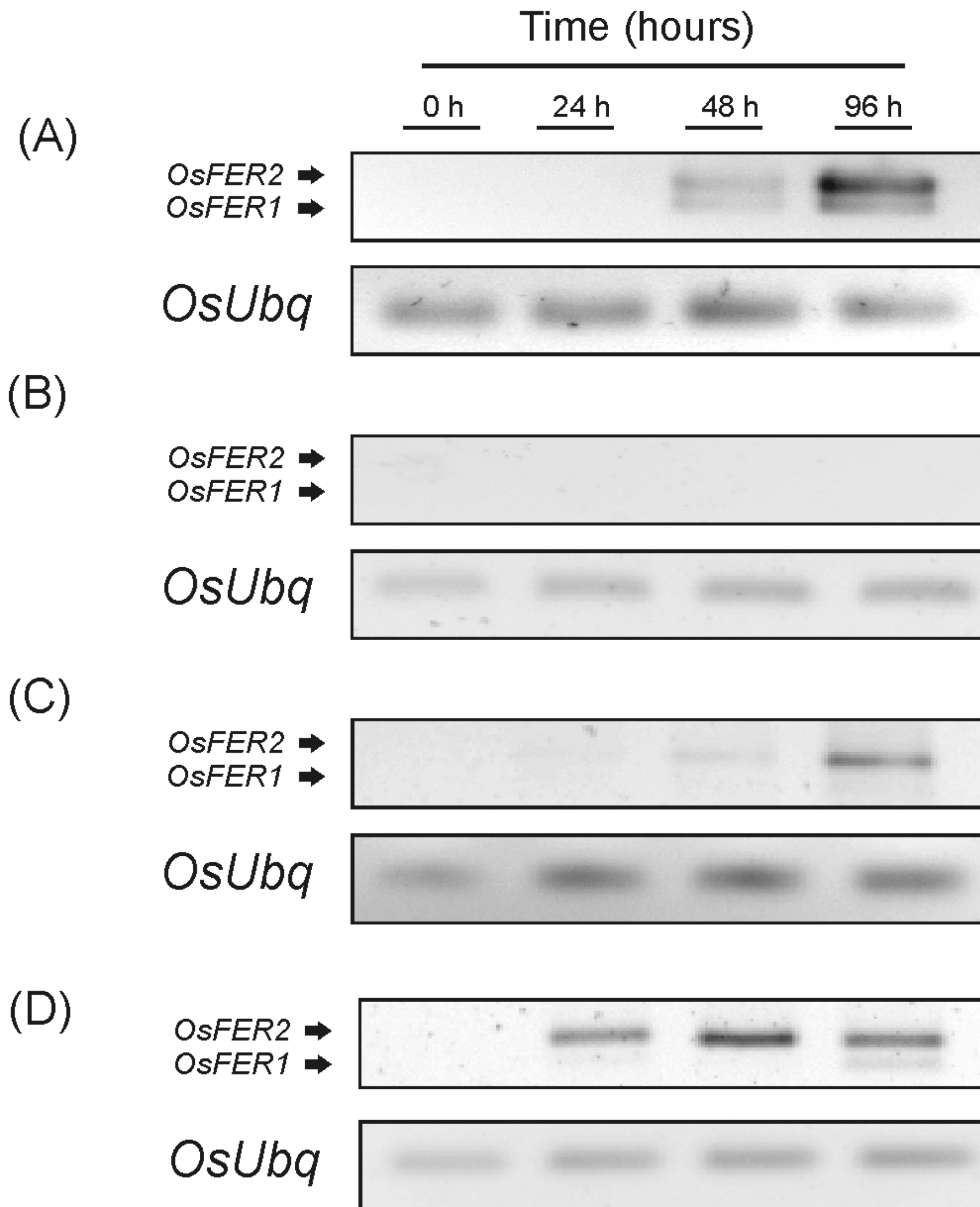
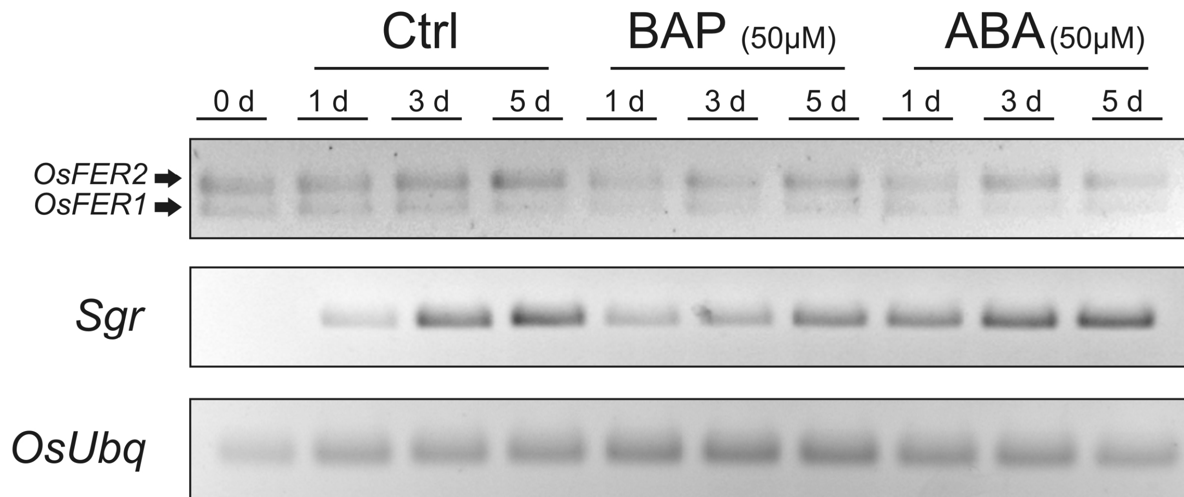


Figure 6



Capítulo 5

“Regulation of iron homeostasis-related genes in rice by iron abundance”

Short Running Title: **Iron regulation of gene expression**

Regulation of iron homeostasis-related genes in rice by iron abundance

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Abstract

Iron is an essential nutrient for virtually all organisms. Due to its transition properties, is part of several essential enzymes in plants. Although abundant in soil, iron is mainly found as insoluble oxides, and thus, plants have evolved distinct strategies for the uptake of iron under low availability. However, in waterlogged and lowland rice, iron excess is a common problem, leading to losses in plant productivity. In this work, we describe the expression profile of twenty iron homeostasis-related genes in rice plants exposed to deficiency, excess and control levels of iron. The expression of the analyzed genes was distinctly altered by the treatments. Interestingly, iron excess increases the relative gene expression of three ZIP rice genes (*OsZIP1*, *OsZIP4* and *OsZIP8*), probably as a result of Zn imbalance due to the presence of iron deposits on the root tissue. Two NRAMP genes (*OsNRAMP4* and *OsNRAMP5*) were also up-regulated by iron excess, suggesting a role of NRAMP genes in response to high iron concentrations. In addition, four other genes belonging to distinct gene families (*OsIRT1* and *OsIRT2*, *OsNRAMP1* and *OsYS7*) showed a similar iron regulation. While the exposure to iron deficiency increased their relative abundance, the opposite regulation was observed in plants exposed to iron excess. The corresponding four proteins (*OsIRT1* and *OsIRT2*, *OsNRAMP1* and *OsYS7*) have been shown in the literature to be Fe-uptake transporters, with strong affinity for this metal. We show that these four genes are responsive to iron in a coordinated way, suggesting the existence of a common regulatory pathway.

Introduction

Iron (Fe) is an essential micronutrient for plants. Due to its capacity to accept and donate electrons, it plays important roles in the growth and development of plants, being necessary in fundamental physiological processes, such as photosynthesis and respiration. Plants require Fe at 10^{-9} to 10^{-4} M for their proper development and reproduction, and, although abundant in the soil, Fe is mainly present as Fe^{3+} oxides, poorly soluble in neutral-to-alkaline soils, being available at 10^{-17} M concentration (Guerinot & Yi, 1994). However, in specific environmental conditions such as waterlogged and lowland cultivated rice, Fe excess can be a severe problem. Fe toxicity is the major nutritional disorder in lowland rice production, caused by the excessive amounts of soluble Fe in the soil solution (Ponnamperuma, 1972). This metal can act as catalyst in the formation of the hydroxyl radical, a potent oxidizing agent, leading to the oxidation of DNA, lipids and proteins (Halliwell & Gutteridge, 1984). Thereby, the regulation of iron homeostasis is crucial for the maintainance of adequate levels of basal metabolic functions, avoiding excessive, toxic levels.

Higher plants have evolved two distinct strategies to capture Fe when its availability is low. One strategy is based on the solubilization of Fe in the rhizosphere (through the extrusion of H^{+}), reduction of Fe^{3+} using a Fe^{3+-} reductase and the uptake of Fe^{2+} though a high affinity transporter (strategy I, used by dicots and non-grass monocots). Grasses developed another strategy (strategy II), based in the secretion of phytosiderophores in the rizhosphere and the subsequent uptake of Fe^{3+}-phytosiderophore complexes. The amount of phytosiderophores secreted in the rizhosphere is increased under Fe limitation, and tolerance to Fe deficiency is thought to be dependent on the amount of phytosiderophores

secreted from plants roots (Marschner *et al.*, 1987). Nicotianamine (NA) is a key intermediate in the biosynthesis of phytosiderophores (Shojima *et al.*, 1990) and Fe deficiency regulates the expression of several genes involved in NA biosynthesis (Higuchi *et al.*, 2001). NA is also important for Fe transport within the plant body, being responsible for delivery of Fe in vegetative and reproductive plant organs (Takahashi *et al.*, 2003).

A large number of transporters that are induced by Fe deficiency have been identified in *Arabidopsis* and in other plant species. As pointed out by Curie et al (2009), the YSL family of transporters represents a serious candidate for the transport of NA-chelates across plant cell membranes. The YSL transporters belong to the oligopeptide transporters family (Yen *et al.*, 2001), firstly identified in maize as a protein that mediates Fe^{3+}-phytosiderophore transport (Curie *et al.*, 2001). Rice has eighteen genes belonging to the yellow stripe-like family (Gross *et al.*, 2003; Koike *et al.*, 2004). Two genes were characterized at molecular and biochemical level, *OsYSL2* (or *OsYS6* – Gross *et al.*, 2003), encoding a rice metal-nicotianamine transporter that may be responsible for the phloem transport of Fe and Mn (Koike *et al.*, 2004), and *OsYSL15* (or *OsYS7* – Gross *et al.*, 2003), a gene induced by Fe deficiency in roots (Koike *et al.*, 2004), which appears to be the dominant Fe^{3+}-deoxymugineic acid transporter in early plant development (Inoue *et al.*, 2008).

Among the other identified genes, the ZIPs (ZRT, IRT-like proteins) appear to be involved in the transport of Fe, Zn, Mn, and Cd, with family members differing in their substrate specificity (Guerinot 2000). In *Arabidopsis*, one gene belonging to this family, *AtIRT1*, is considered the major transporter for high affinity Fe uptake in roots (Vert *et al.*, 2002). Rice has thirteen identified ZIP genes (Gross *et al.*, 2003), including two very similar to *AtIRT1*, named *OsIRT1* (Bughio *et al.*, 2002) and *OsIRT2* (Gross *et al.*, 2003). Both genes were

shown to reverse the growth defects of yeast Fe-uptake mutants, confirming their predictable Fe-uptake properties (Bughio *et al.*, 2002; Ishimaru *et al.*, 2006). It is interesting to note that rice, although being a strategy II plant for Fe uptake, also possesses strategy I genes, being capable of absorb iron as Fe^{3+}-phytosiderophore and as Fe^{2+}, although not using a Fe^{3+}-reductase (Ishimaru *et al.*, 2006).

Another class of metal transporters is encoded by the NRAMP (natural resistance-associated macrophage protein) gene family, composed of seven members in *Arabidopsis* (Thomine *et al.*, 2000) and eight members in rice (Gross *et al.*, 2003). The NRAMP genes are widely distributed throughout living organisms and are involved in the transport of a broad range of divalent metal cations, including iron (Gunshin *et al.*, 1997). Expression studies in plant tissues indicated that while *OsNRAMP1* is expressed primarily in roots, and *OsNRAMP2* is primarily expressed in leaves, *OsNRAMP3* is expressed in both tissues (Belouchi *et al.*, 1997). *OsNRAMP2* expression is strongly induced one day after sowing (Nozoye *et al.*, 2007) suggesting a possible role of NRAMP genes in the remobilization of metals during rice seed germination.

Apart the identification of genes involved in Fe uptake, few data about the effects of high iron concentration or deficiency on the regulation of these Fe-homeostasis related genes are available. In this work, we describe the expression profile of twenty Fe homeostasis-related genes (five YS, eight ZIPs, and seven NRAMPs) in leaves and roots of rice plants exposed to excess, deficiency and control iron concentrations.

Materials and methods

Plant material, growth and treatments

Rice grain (*Oryza sativa* L. ssp. *japonica*) from cultivar Nipponbare were surface sterilized in $NaClO_4$ 2.5% for 10 minutes followed by ethanol 70% for 15 minutes and washed in abundant distilled water. Seeds were germinated in Petry dishes and seedlings were kept in the dark during the first 48 hours, then they were transferred to 16h/8h day light regime at 28°C for two days and were transferred to pots with vermiculite and watered with nutrient solution (Yoshida, 1981). After 10 days, plants were transferred to hydroponic conditions, using the same nutrient solution, and after an adaptation period (10 days) plants were subjected to different concentrations of Fe: Fe excess (9 mM), control (0.15 mM), both using $FeSO_4$ as the Fe source, and deficiency (without Fe and adding 0.15 mM ferrozine – a free iron chelator)**.** Plants (roots and leaves) were collected after 0, 24 and 96 hours of exposure to treatments, immediately frozen and stored at -80ºC until further analyses.

RNA isolation and cDNA synthesis

Total RNA was isolated from plant material (leaves and roots) using Trizol (Invitrogen) following the manufacturer's instructions. RNA integrity was confirmed in 1.6% agarose gels and RNA purity estimated from the absorbance ratio at 260 and 280 nm. RNA was quantified using the Quant-iT RNA Assay Kit and the Qubit Fluorometer (Invitrogen). One microgram of total RNA was treated with DNAse (Invitrogen) and reverse transcribed using M-MLV (Invitrogen) and oligo-dT(30). All cDNAs were diluted (1:100) in water and readily used for real-time PCR analyses (qPCR).

qPCR conditions and analysis

Polymerase chain reactions were performed in an ABI 7300 (Applied Biosystems), using SYBR® Green (Invitrogen) to monitor dsDNA synthesis. The following standard thermal profile was used for all PCRs: 95°C for 5 min; 40 cycles of 95°C for 15 sec, 60-68°C for 10 sec, 72°C for 15 sec and 60°C for 35 sec. The PCR efficiency from the exponential phase (E) was calculated for of each individual amplification plot using the equation $(1+E)=10^{slope}$ (Ramakers *et al.*, 2003). Data from each reaction was normalized by the corresponding E value. In order to compare data from different PCR runs or cDNA samples, C_t values for all genes were normalized to the C_t value of *Ubq* (Miki *et al.*, 2005) using the equation $Y = 2^{-\Delta Ct}$ ($\Delta Ct = Ct_{ubq} – Ct_{target\ gene}$). The average C_t value for *OsUbq* was 18.08 (±0.68) for all plates/templates measured in this series of experiments. Primers corresponding to all tested genes are listed in Table 1

Results

Iron regulation of ZIP gene family

The expression pattern of both IRTs genes (*OsIRT1* and *OsIRT2*) was similar, although with higher abundance of the *OsIRT1* transcripts (Figure 1). Both genes are expressed only in roots, are up-regulated by Fe deficiency, and down-regulated upon Fe excess.

Exposure to Fe excess also altered the expression pattern of the ZIP genes in rice (Figure 2), resulting in higher accumulation of *OsZIP1* in leaves and of *OsZIP7* and *OsZIP8* transcripts in roots. The studied ZIP genes also showed a distinct expression pattern in roots and leaves, with *OsZIP4*, *OsZIP5*, *OsZIP6* and *OsZIP8* being more expressed in roots (Figure 2).

NRAMP gene family

Among the eight NRAMP genes studied, three were regulated by Fe (Figure 3). Root expression of Os*NRAMP1* was up-regulated by exposure to Fe deficiency, readily after 24 hours, and down-regulated (also after 24 hours) by high Fe concentration. Two genes, Os*NRAMP4* and Os*NRAMP5*, were up-regulated in roots after 96 hours of exposure to Fe excess.

Expression of *OsNRAMP1*, *OsNRAMP3*, *OsNRAMP4*, *OsNRAMP5* and *OsNRAMP7* was higher in roots than in leaves. *OsNRAMP6* was the only gene with higher expression in leaves than in roots (Figure 3). We were not able to detect any amplification signal of *OsNRAMP8*, neither in roots or leaves.

Yellow Stripe-Like gene family

Expression of *OsYS7* was increased by the Fe deficiency treatment and reduced by Fe excess, readily after 24 hours. Also, the relative abundance of *OsYS2*, *OsYS4* and *OsYS7* mRNAs was higher in roots, whereas *OsYS3* and *OsYS8* expression was higher in leaves (Figure 4).

Discussion

As expected, exposure to distinct Fe concentrations led to differential expression of members of the NRAMP, ZIP and YS families in leaves and roots of rice plants. Among the ZIP genes studied, the expression profile of *OsIRT1* and *OsIRT2* showed a clear Fe regulation, with Fe deficiency inducing the expression of both genes in roots, and Fe excess inhibiting the *OsIRT1* and *OsIRT2* transcript accumulation in roots. Both genes are able to

complement Fe-uptake-defective yeast mutants, and are thought to be essential Fe^{2+} transporters (Ishimaru *et al.*, 2006).

Among the other tested ZIP genes, three genes (*OsZIP1*, *OsZIP7* and *OsZIP8*) were up-regulated by Fe excess, although with distinct organ specific abundance. The negative effects of Fe excess in Zn uptake is a known process, resulted from a physical impediment caused by the accumulation of Fe deposits in the root apoplast, induced by the root oxidative capacity (Zhang *et al.*, 1999). Probably this limitation is capable to induce Zn deficiency, and thus, the up-regulation of the three ZIP genes could be a response to limitations in Zn uptake, caused by the excessive accumulation of Fe deposits on roots.

Fe excess also induced the expression of *OsNRAMP4* and *OsNRAMP5*, suggesting a possibly role of both genes in the responses against high levels of Fe. Both genes were mainly expressed in roots. It is known that one of the physiological responses to Fe excess is its accumulation in the vacuole (Becker *et al.*, 1998). In *Arabidopsis*, two NRAMP genes (*AtNRAMP3* and *AtNRAMP4*), are essential for the vacuolar Fe transport (Lanquar *et al.*, 2005), but whether *OsNRAMP4* and *OsNRAMP5* are involved in the storage of excessive Fe in rice vacuoles remains to be tested.

Interestingly, the expression profile of *OsNRAMP1* also showed a clear Fe regulation, similar to *OsIRT1* and *OsIRT2*, with Fe deficiency inducing the expression and Fe excess inhibiting its mRNA accumulation in roots. In tomato, *LeNRAMP1* plays an essential role in the mobilization of Fe in the vascular parenchyma upon Fe deficiency (Bereczy *et al.*, 2003). Considering that *OsNRAMP1* is capable to complement yeast mutants defective in Fe-transport (Curie *et al.*, 2000), a similar function could be attributed to *OsNRAMP1*. Thus, the observed down-regulation in *OsNRAMP1* expression could be an attempt to lessen the Fe arrival in the leaf tissue. It is known that citrate is the principal Fe chelator in

the xylem (Cataldo *et al.*, 1988), and a Fe-citrate transport (*OsFRDL1*) was recently described (Yokosho *et al.*, 2008). It will be interesting to analyze the effects of Fe excess in the expression of *OsFRDL1* and in the regulation of Fe mobilization and translocation to the leaves.

The *OsYS7* gene was clearly Fe-regulated, with induction of its relative expression by exposure to Fe deficiency and inhibition by Fe excess. The expression of *OsYS7* is known to be induced by Fe deficiency (Koike *et al.*, 2004), and it was recently shown that *OsYS7* can complement a yeast mutant defective in Fe uptake. This complementation was possible only when Fe was supplied as Fe^{3+}-deoxymugineic acid. The same worked showed that the *OsYS7* protein is localized at the plasma membrane (Inoue *et al.*, 2008). Thereby, the Fe excess down-regulation observed in our results could be a response to the high levels of Fe in the nutrient solution.

Interestingly, a similar coordinated response to the variable Fe concentrations could be observed in genes that belong to distinct gene families – *OsIRT1* and *OsIRT2*, *OsNRAMP1* and *OsYS7*. The capacity to restore the high affinity Fe uptake in yeast is a common feature of these genes, which are able to transport Fe^{2+} (in the case of *OsIRT1*, *OsIRT2* and *OsNRAMP1*) and Fe^{3+}-deoxymugineic acid (in *OsYS7*). This coordinated response could suggest that a common pathway regulates their repression. Fe excess is known to cause an oxidative burst in the chloroplast, an early event in the signal transduction involved in the expression of *AtFer1* (Arnaud *et al.*, 2006), a ferritin protein known to be involved in the Fe excess response. In the case of *AtFer1*, a *cis*-acting element named IDRS (Iron-dependent regulatory sequence) responsible for the transcriptional repression of *AtFer1* and *ZmFer1* under low iron supply was described (Petit *et al.*, 2001). A similar regulatory pathway could operate in the Fe excess induction of the *OsIRT1*, *OsIRT2*, *OsNRAMP1* and

OsYS7 genes in rice roots, although in the opposite direction (induction instead of repression under low Fe supply). The existence of common regulatory elements in the promoters of these genes needs to be investigated, as well as the nature of a possible transcription factor that could bind to these elements.

As a strategy I plant, rice is supposed to induce the expression of Yellow Stripe genes under Fe deficiency, what actually happens. However, the induction of a typical strategy II gene, *OsIRT1*, has also been reported (Ishimaru *et al*., 2006), and was confirmed by this work. It was suggested that the capacity to absorb Fe^{2+}, provided by the two IRT genes, is advantageous for growth in submerged conditions (Ishimaru *et al*., 2006), present for most of the world rice production. Due to the anaerobic and reductive environment created by waterlogging, large amounts of Fe^{2+} can be solubilized from the soil (Ponnamperuma, 1972). Indeed, Fe toxicity is a common problem to rice cultivated in such conditions. Our results showing that both rice IRT genes are negatively regulated by Fe excess could be indicative that Fe transport from the soil solution in waterlogged environments is not performed by IRT proteins, but rather by unknown transporters, probably with low affinity for the metal.

Acknowledgements

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Table 1. Gene-specific primers used for qPCR		
Gene	**Primer Sequence (5' – 3')**	**Product size (bp)**
OsIRT1	GCAATTCGCTGCATTGTTAG GAAGTACATCATCAGTCACGAA	162
OsIRT2	GTCCGTCATGGCCAAGTG GGATGATGATCCGTACGGCAGAAG	145
OsZIP1	GCTCATGATTTCGGACGTTT CCCAAAATCCATGGAAACAA	177
OsZIP4	GCATAGATCTTCAGAATAACAGAGGA TCACCTGAGATAAGCTTTGGTTT	156
OsZIP5	GATTCTTGGGCAAATGGTGT ACAACGCTGGGGATTATTTG	258
OsZIP6	GACGATGACGATGAGGGTTT CACGAGGAGGAGGAAGCTC	274
OsZIP7	GCGAAAGCAACAGTGATCATGGCGACTTTC GCAGCTCTTGGTTGCTCTGAAGATCTCATG	187
OsZIP8	CAGGAATGGCAGGTTTTTGT AGTTTCAACCAACGGAGTGG	113
OsNRAMP1	CGGTGTTGGCTGGTTTTTAT CATTCTGCCAATCTGCCAAT	193
OsNRAMP2	GCCTGCTTGTATTAGCTCCAG GGAGAAGCCCAACCAATTC	174
OsNRAMP3	GTTAGCCAGCTGATCCCTCA AGAAGCTTTGGTGTCACAGGA	161
OsNRAMP4	TTGCTTGCTGAGTAGTGCAT GCTGCTTAGAAACAACAACAAGAA	126
OsNRAMP5	GTCGGAGCCGTTCGTTTAT GGCTCTGCCCTGAATTATGA	159
OsNRAMP6	GCTCAAAGCCTCGAAATCAT TGGCGTGGAAGAGAATTTTA	125
OsNRAMP7	GCTGCCAAATCAGATCATCA GCTTCAGGACGACACAGTCA	240
OsYS2	TCTTGATCGAGGAAGAAGTGG TGCCATAGTATGTTCGTTGGA	162
OsYS3	CTCAAGCTAGCCTTCCATCG TGCTACACCAGCTGCTTCTC	312
OsYS4	AAGTTGGACGCCTTCTTGG CAGTTTGCCACGACTCCTAA	153
OsYS7	GGATTGCAGAAATAAACAGTGATG′ TGCCAAACTAAACAATTCTCAA	167
OsYS8	TGTGCATGTACTTCAAGCCATC AAGAACAAAGTTACTGCACTTTTGC	150

Figure Legends

Figure 1. Expression profile of the *OsIRT1* and *OsIRT2* genes in roots and leaves of rice plants exposed for 0, 24 and 96 hours to control (Ctrl), Fe deficiency (Fe^-) or Fe excess (Fe^+) treatments. Values represent the mean ± standard error of three biological replicates (n=3). Expression values are given in relation to the rice ubiquitin (*OsUbq)* gene expression and normalized in relation to the PCR efficiency from the exponential phase (*E*) from each individual amplification.

Figure 2. Relative gene expression of the rice ZIP genes *OsZIP1*, *OsZIP4*, *OsZIP5*, *OsZIP6*, *OsZIP7*, and *OsZIP8* in roots and leaves of rice plants exposed for 0, 24 and 96 hours to control (Ctrl), Fe deficiency (Fe^-) or Fe excess (Fe^+) treatments. Values represent the mean ± standard error of three biological replicates (n=3). Expression values are given in relation to the rice ubiquitin (*OsUbq)* gene expression and normalized in relation to the PCR efficiency from the exponential phase (*E*) from each individual amplification.

Figure 3. NRAMP relative gene expression (*OsNRAMP1*, *OsNRAMP2*, *OsNRAMP3*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6* and *OsNRAMP7*) in roots and leaves of rice plants exposed for 0, 24 and 96 hours to control (Ctrl), Fe deficiency (Fe^-) or Fe excess (Fe^+) treatments. Values represent the mean ± standard error of three biological replicates (n=3). Expression values are given in relation to the rice ubiquitin (*OsUbq)* gene expression and normalized in relation to the PCR efficiency from the exponential phase (*E*) from each individual amplification.

Figure 4. Expression profile of the genes *OsYS2*, *OsYS3*, *OsYS4*, *OsYS7* and *OsYS8* in roots and leaves of rice plants exposed for 0, 24 and 96 hours to control (Ctrl), Fe deficiency (Fe^-) or Fe excess (Fe^+) treatments. Values represent the mean ± standard error of three biological replicates (n=3). Expression values are given in relation to the rice ubiquitin (*OsUbq)* gene expression and normalized in relation to the PCR efficiency from the exponential phase (*E*) from each individual amplification.

Figure 1

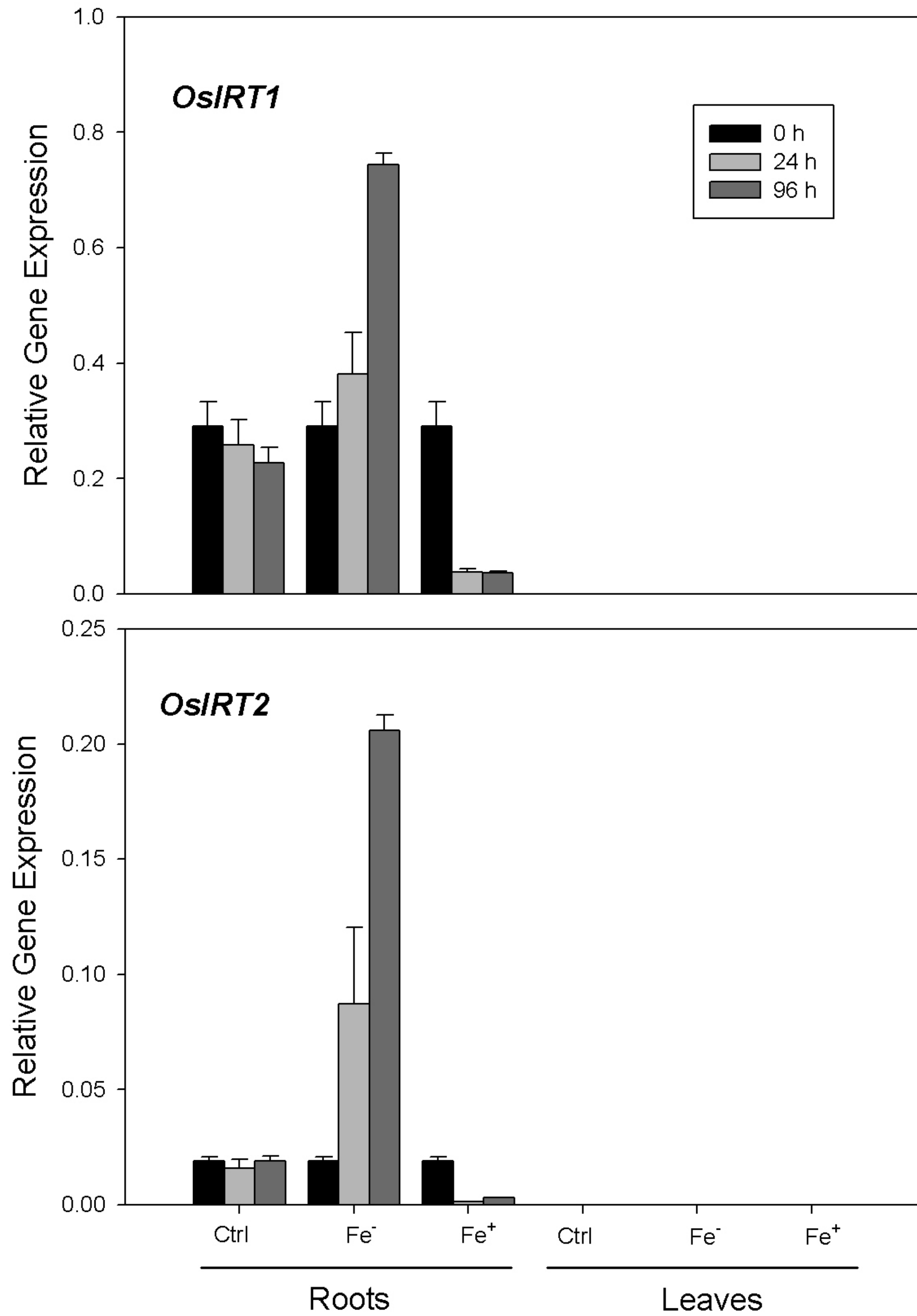


Figure 2

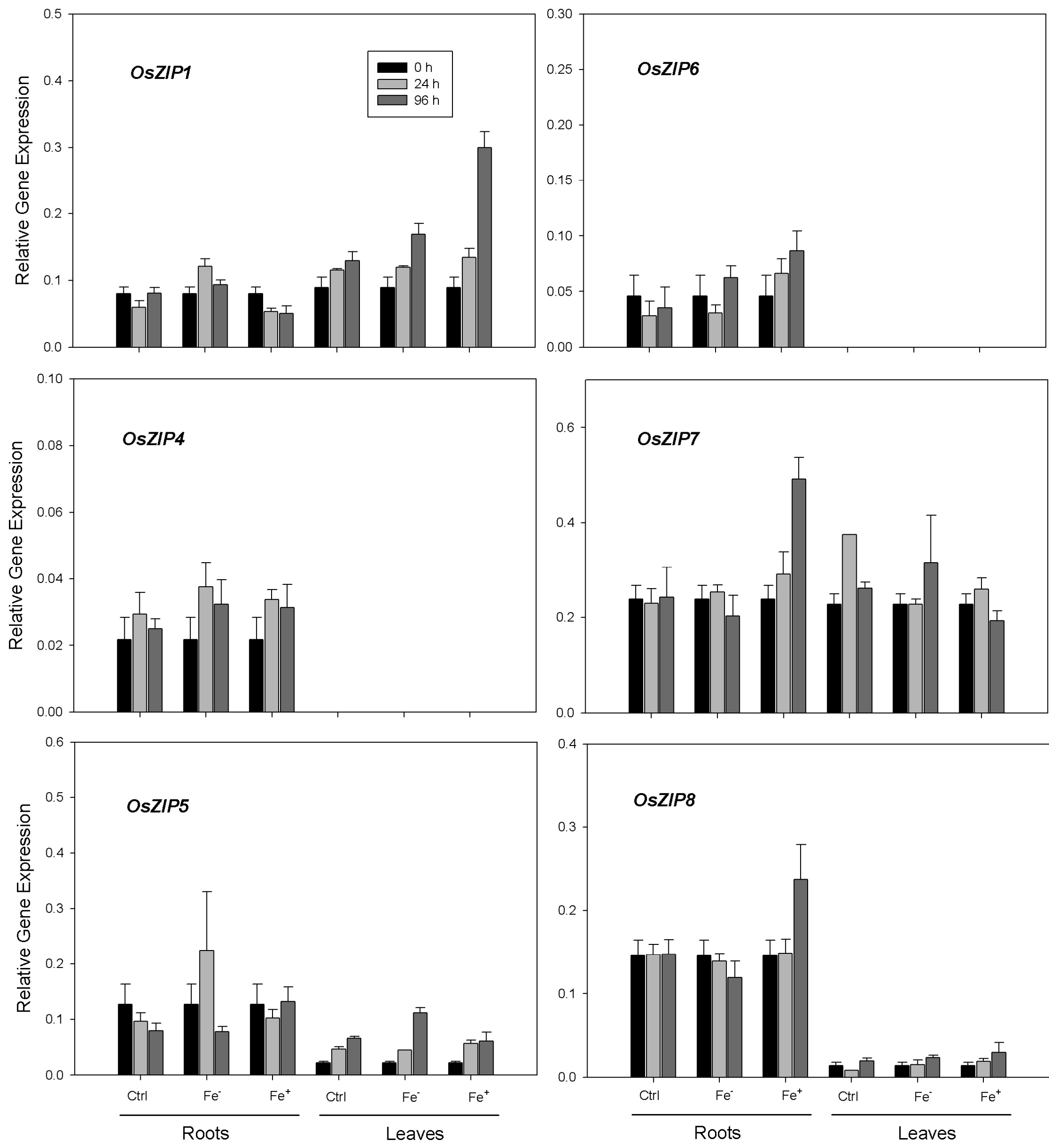


Figure 3

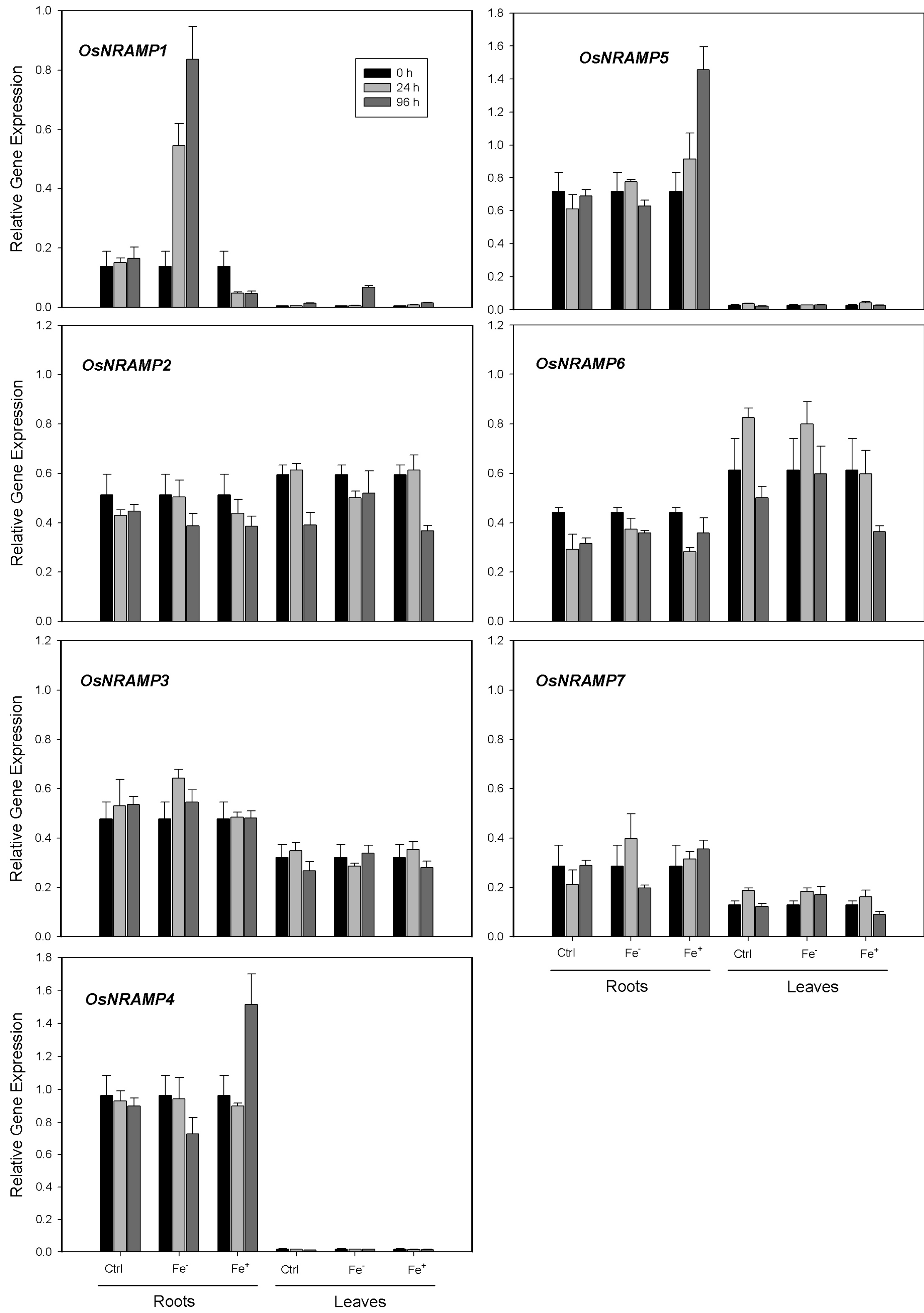
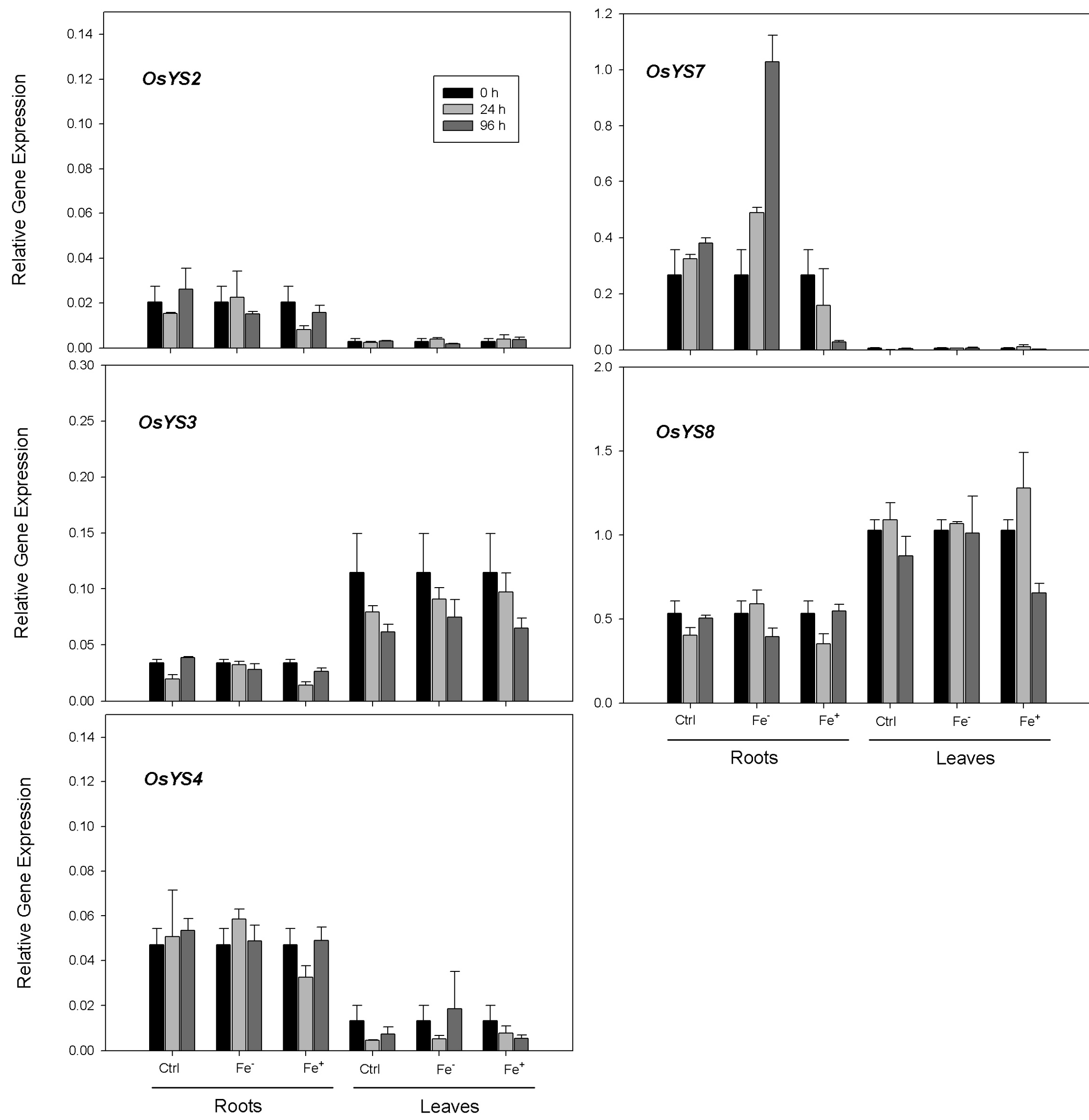


Figure 4



Considerações Finais

Utilizando diversas abordagens foi possível analisar os efeitos do excesso ferro em diferentes genótipos de arroz, seja em plantas cultivadas a campo ou crescidas em laboratório. De maneira geral, o excesso de ferro apresentou um evidente efeito foto-oxidativo, levando a uma diminuição no crescimento, degradação de clorofila e a danos oxidativos. Os efeitos tóxicos do ferro foram exclusivos a plantas sensíveis, confirmando a classificação das cultivares realizada pelo IRGA e assim, validando nossas abordagens experimentais. O efeito tóxico observado deveu-se ao acúmulo de grandes níveis de ferro em tecidos vegetais, não tendo sido causado pela deficiência de outros nutrientes, caracterizando assim uma toxidez direta.

Em folhas, a maior parte do ferro encontra-se nos cloroplastos (Terry & Low, 1982); logo, qualquer reação adversa causada pelo acúmulo de níveis excessivos do metal inicialmente ocorre nessa organela. Como observado (Capítulo 3), o excesso de ferro levou a uma drástica queda na atividade fotossintética, diminuição da sua taxa máxima de carboxilação, capacidade de transporte de elétrons, entre outros efeitos negativos. Estes dados sugerem que os efeitos tóxicos podem estar diretamente associados a um distúrbio no metabolismo cloroplastídico, afetando o metabolismo de carbono.

As cultivares tolerantes diferiram quanto ao acúmulo de ferro em suas folhas e raízes. A sua localização parece ser uma questão nevrálgica na capacidade e na forma de lidar com o metal. Baseado nisso, podemos dividir as cultivares tolerantes em dois principais grupos:

(I) plantas tolerantes que acumulam ferro em concentrações inferiores às plantas sensíveis;

(II) plantas tolerantes que acumulam ferro em níveis iguais ou superiores às plantas sensíveis.

A capacidade de reter ou de limitar a absorção de ferro é conhecida como um mecanismo utilizado por algumas plantas para evitar seu acúmulo excessivo em partes aéreas, uma capacidade ausente ou limitada em plantas intolerantes à submersão (Wheeler *et al.*, 1985). Entretanto, em contraste com a oxidação de ferro em espaços apoplásticos da raiz (levando à formação de uma placa férrica), plantas da cultivar EPAGRI 108 acumularam grandes quantidades de ferro no simplasto de suas raízes (como observado na Figura 3C, Capítulo 2). Porém a mesma cultivar, quando crescida em laboratório, não apresentou esse comportamento, acumulando baixas concentrações de ferro em raízes tratadas ou não com a solução DCB (solução responsável pela solubilização do ferro precipitado em espaços apoplásticos da raiz). Plantas cultivadas a campo apresentam uma prolongada e gradual exposição a altos níveis de ferro, sendo que o máximo de disponibilidade do metal ocorre após 30 dias de submersão do campo (Ponnamperuma *et al.*, 1972). Enquanto isso, plantas crescidas em laboratório foram submetidas a uma grande concentração de ferro por um período mais curto (nove dias) de exposição aos tratamentos. Porém, em ambas as condições, plantas da cultivar EPAGRI 108 não apresentaram sintomas de toxidez, apresentando-se como tolerantes. Assim, essas diferentes respostas podem ser relacionadas a uma distinta capacidade de limitar a captura do metal.

A habilidade de regular a captura de ferro e impedir seu acúmulo pode igualmente ser considerada como um mecanismo de tolerância. Como observado em plantas da cultivar EPAGRI 108 crescidas em laboratório, esta habilidade pode estar relacionada com o remodelamento e a lignificação das paredes celulares de suas raízes. A endoderme é primeira barreira seletiva na absorção ativa de nutrientes, e a sua lignificação pode atuar

como um impedimento físico para a absorção de grandes quantidades de ferro. Igualmente, o acúmulo de ferro em vacúolos já foi sugerido como um mecanismo de tolerância usado por plantas expostas a altos níveis de ferro (Becker *et al.*, 1998), impedindo assim a translocação de altos níveis de ferro das raízes para as folhas.

Vários genes codificadores de transportadores vacuolares de ferro já foram descritos em plantas (Briat *et al.*, 2007), e dois genes pertencentes à família gênica NRAMP (*OsNRAMP4* e *OsNRAMP5*) tiveram suas expressões relativas aumentadas, o que pode sugerir seu envolvimento em respostas a altas concentrações de ferro (como observado na Figura 3, Capítulo 5). A análise da expressão destes e de outros genes (e.g. CC1, similar a *AtVIT1* identificado no Capítulo 3) em outras cultivares de arroz, bem como a sua caracterização funcional, devem elucidar os seus papéis em mecanismos utilizados pelas plantas para tolerar o excesso de ferro.

Em contrapartida, plantas tolerantes que acumulam ferro em níveis iguais ou superiores às plantas sensíveis podem fazer uso de distintos mecanismos. Dentre eles, a capacidade de acumular ferro em espaços simplásticos de suas folhas (em níveis semelhantes aos observados em plantas sensíveis) parece ser de central importância para a cultivar IRGA 420 (Capítulo1).

Além disso, a capacidade de detoxificação dos danos causados por excessivos níveis de ferro também tem sido considerada um mecanismo de tolerância ao excesso de ferro em arroz (Wu *et al.*, 1998). Embora esta capacidade tenha sido observada em plantas da cultivar EPAGRI 109, o detalhamento das respostas antioxidantes foi essencial para a definição da sua real participação em mecanismos de tolerância. Enquanto a enzima APX parece estar envolvida diretamente com os danos causados pelos altos níveis de ferro, tendo sua atividade aumentada em plantas sensíveis, a participação da enzima SOD parece estar

envolvida diretamente na capacidade de plantas da cultivar EPAGRI 109 tolerar altos níveis do metal, juntamente com uma maior atividade de GR e DHAR. Interessantemente, a maior atividade de CAT comum às plantas tolerantes e sensíveis pode indicar uma resposta comum dos genótipos frente ao excesso de ferro.

A exposição de plantas ao excesso de ferro também foi responsável pela indução da expressão dos genes de ferritina em arroz (*OsFER1* e *OsFER2*) (detalhado no Capítulo 4). Essa indução mostrou-se dependente de uma etapa oxidativa, indicando que o metal *per se* não foi capaz de regular a expressão de ferritina. Esse resultado sugere, assim como observado por Ravet *et al*. (2008), a atuação de ferritinas como uma resposta a danos mediados pelo ferro e não somente como um estoque celular do metal.

Neste trabalho, o impacto de altos níveis de ferro foi explicitado em diferentes genótipos de arroz, seja em plantas crescida a campo ou em laboratório. Além disso, foi possível propor novos mecanismos, bem como o detalhamento de mecanismos previamente propostos na literatura. A definição das respostas de diferentes genótipos de arroz frente a altos níveis de ferro pode auxiliar no melhoramento da cultura, bem como na melhor compreensão da homeostase de ferro em plantas de arroz.

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