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ESTUDOS SOBRE AS PROTEÍNAS FERRITINA E OsNRAMP7 EM PLANTAS DE ARROZ (*Oryza sativa* L.)

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There will come a time when you believe everything is finished.
Yet that will be the beginning.

Louis L'Amour

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Resumo

O arroz é um dos cereais mais produzidos e consumidos no mundo, cultivado em aproximadamente 156 milhões de hectares, com uma produção mundial de mais de 600 milhões de toneladas por ano. O arroz é, hoje, alimento básico para mais de dois terços da população mundial. Contudo, minerais como ferro e zinco são perdidos durante o processo de beneficiamento dos grãos para comercialização. Uma vez que a deficiência de ferro afeta cerca de três bilhões de pessoas e é a deficiência mineral mais comum em humanos, diversos esforços têm sido feitos para aumentar a concentração deste mineral em grãos de arroz. Diversos projetos têm como objetivo compreender o mecanismo de translocação de nutrientes para grãos de arroz, visando o aumento de sua concentração com fins de biofortificação do alimento. Para melhor compreender a homeostase de ferro em plantas de arroz, conduzimos experimentos para analisar possíveis funções de duas proteínas. Proteínas da família NRAMP (Natural Resistance Associated Macrophage Protein) foram descritas como tendo envolvimento na homeostase de ferro em diferentes organismos. OsNRAMP7 apresenta propriedades características da família, como os motivos DPGN e MPH, possivelmente envolvidos no transporte de metais. Oócitos de *Xenopus* injetados com o mRNA de *OsNRAMP7* apresentaram aumento significativo na concentração de ferro. A expressão heteróloga da proteína em oócitos indica o envolvimento da proteína no transporte transmembrana de ferro. Ferritina é outra proteína envolvida na homeostase de ferro nas células. Ferritinas são proteínas esféricas, capazes de armazenar ferro no seu interior, agindo também como um estoque de ferro nas células. O armazenamento de ferro dentro desta proteína pode prevenir reações que levam a produção de radicais livres e, consequentemente, estresse oxidativo. Duas cópias do gene da ferritina foram descritas em arroz. Respostas ao estresse oxidativo em uma linhagem mutante de arroz para o gene *OsFER2* foram estudadas. Quando submetidas a excesso de ferro, plantas mutantes tiveram aumento na concentração de MDA (malondialdeído) nas partes aéreas e da atividade da enzima APX (ascorbato peroxidase) em raízes, revelando respostas ao dano oxidativo quando há baixa produção de ferritina. Plantas mutantes acumulam menos biomassa do que plantas WT (wild type) mesmo em condição controle de crescimento. Isso pode indicar um possível papel da ferritina na homeostase de ferro em plantas de arroz, ainda que as mesmas não estejam em estresse por excesso de ferro. Mecanismos compensatórios como o aumento da quantidade da proteína

frataxina e aumento do influxo de ferro para vacúolos também devem ser investigados. Mais experimentos são necessários para melhor compreensão do papel da ferritina na homeostase de ferro em arroz. Não obstante, com os experimentos aqui apresentados é possível determinar o envolvimento da proteína OsNRAMP7 na homeostase de ferro em arroz.

Palavras-chave: *Oryza sativa*, ferro, *OsNRAMP7*, *Xenopus*, ferritina, estresse oxidativo.

Abstract

Rice is one of the most produced and consumed cereals in the world, cultivated in approximately 156 million hectares, with a world production of over 600 million tons. It is a staple food for two thirds of the world population. However, minerals such as iron and zinc are lost during rice processing for commercialization. Since iron deficiency affects around three billion people, and is the most common mineral deficiency in humans, several efforts have been made in order to increase this nutrient's levels in rice grains. Several projects have as goal to understand translocation mechanisms of nutrients to rice grains as to increase their levels for biofortification purposes. To better understand iron homeostasis in rice plants, we conducted experiments in order to analyze the putative role of two proteins. The NRAMP (Natural Resistance Associated Macrophage Protein) family was described as having an important role in iron homeostasis in different organisms. OsNRAMP7 presents characteristic features of the family, as motifs DPGN and MPH, said to be involved in metal transport. *Xenopus* oocytes injected with *OsNRAMP7* mRNA exhibited a significant increase in iron content. Heterologous expression of the protein in oocytes indicated that the protein is involved in transmembrane iron transport. Ferritin is another protein involved in intracellular iron homeostasis. Ferritins are spherical proteins capable of storing iron in their core, also acting as an iron buffer in cells. Storage of free iron inside this protein may prevent reactions that lead to the formation of oxygen radicals and, therefore, to oxidative stress. Two ferritin genes have been described in the rice genome. We studied the oxidative stress response of a mutant line of rice with impaired expression of *OsFer2*. When subjected to iron excess, mutant plants increased MDA (malondialdehyde) concentration in shoots and APX (ascorbate peroxidase) enzyme activity in roots, revealing oxidative damage responses when ferritin production is impaired. Mutant plants have lower weight than WT (wild type) even in control growth condition. This may indicate a possible role of ferritin in iron homeostasis in rice plants, even when they are not under iron stress. Compensative mechanisms such as increase of frataxin levels and iron influx to the vacuole should be investigated. More experiments are required for a proper understanding of ferritin role in iron homeostasis. Still, with these experiments allowed to determine the involvement of the OsNRAMP7 protein in iron homeostasis in rice.

Keywords: *Oryza sativa*, iron, *OsNRAMP7*, *Xenopus*, ferritin, oxidative stress.

Lista de abreviaturas

ABC – ATP Binding Cassete

APX – Ascorbate Peroxidase

AtFH – *Arabidopsis thaliana* frataxin

BSA – Bovine Serum Albumin

CAT – Catalase

cDNA – Complementary Deoxyribonucleic Acid

CTM – Consensus Transport Motif

DMA – 2'-Deoxymugineic Acid

DNA – Deoxyribonucleic Acid

DW – Dry Weight

EDX – Energy Dispersive X-ray

Et0/ABS – Quantum Yield of Electron Transport

Et0/TRO – Efficiency with which an electron can move to the PSI electron acceptor

FRD – Ferric Reductase

FRDL – Ferric Reductase Like

FRO – Ferric Chelate Reductase

FST – Flanquing Sequence Tags

Fv/Fm – Maximal Efficiency of PSII Photochemistry

FW – Fresh Weight

GFP – Green Fluorescent Protein

IRE – Iron Regulatory Elements

IRT – Iron Regulated Transporter

ITP – Iron Transport Protein

LEA – Late Embryogenesis Abundant

MA – Mugineic Acid

MATE – Multidrug and Toxic Compound Extrusion Transporter

MBS – Modified Barth's Saline

MDA – Malondialdehyde

MIR – Mitochondrial Iron-Regulated

mRNA – Messenger Ribonucleic Acid

NA – Nicotianamine

NADPH – Nicotianamide Adenine Dinucleotide Phosphate

NRAMP – Natural Resistance Associated Macrophage Protein

OsFER – Oryza sativa Ferritin

OsUbq – Oryza sativa Ubiquitin

PCR – Polymerase Chain Reaction

PETIS – Positron-Emitting Tracer Imaging System

PIC1 – Permease In Chloroplast 1

PIC1ox – Permease In Chloroplast 1 overexpressing lines

PMSF – Phenylmethylsulphonylfluoride

PS – Phytosiderophores

PSII – Photosystem II

QTL – Quantitative Trait Loci

RGRC – Rice Genome Resource Center

RNA – Ribonucleic Acid

RNAi – RNA Interference

ROS – Reactive Oxygen Species

RT-PCR – Reverse Transcription Polymerase Chain Reaction

SOD – Superoxide Dismutase

SPR – Short Postembryonic Root

TBARS – Thiobarbituric Reactive Species

TCA – Trichloroacetic Acid

TMD – Transmembrane Domain

WT – Wild Type

YSL – Yellow Stripe Like

Sumário

Introdução

O arroz é um dos cereais mais produzidos e consumidos no mundo, sendo cultivado em aproximadamente 150 milhões de hectares, com produção mundial de 610 milhões de toneladas em 2004 (IRRI, 2007), servindo assim, de alimento básico para dois terços da população do planeta (Guidolin, 1993).

O arroz, *Oryza sativa L.*, é uma planta anual, da família Poaceae, da qual são conhecidas diferentes variedades cultivadas. Segundo Terres (1998), o arroz cultivado, embora pareça ter sido originado de uma forma perene, é considerado uma gramínea semi-aquática anual. O caule é um colmo, formado por nós e entrenós. De cada nó surge uma folha. A estrutura básica vegetativa é o fitômero, o qual é constituído por um entrenó, um nó, uma folha e uma gema. Freqüentemente, os entrenós basais são muito curtos, proporcionando uma maior concentração de folhas na base da planta. As folhas envolvem o colmo e, têm disposição alternodística e constam de bainha, lígula e lâmina. A bainha é a parte alongada da folha, em forma de cartucho envolvendo o colmo, que nasce em cada nó e, assim, recobre o entrenó. A lígula se constitui na estrutura membranosa situada na face adaxial da folha, na região limítrofe entre a bainha e a lâmina, a qual é linear, paralelinérvia (Boldrini et al., 2005). A inflorescência do arroz é uma panícula de espiguetas, constituídas de um par basal de brácteas estéreis, denominadas glumas, e um eixo denominado ráquila, o qual sustenta as glumas e os antécios (composto por pálea e lema). As glumas e os antécios têm disposição alterno-dística sobre a ráquila. A flor é bissexuada, constando assim dos órgãos sexuais, androceu e gineceu, e de um perianto rudimentar, representado pelas lodículas. É protegida por duas brácteas, a pálea e a lema, que constituem o conjunto chamado antécio. O androceu consta de seis estames e o ovário é bicarpelar, unilocular, unisseminado, com estilete curto e dois estigmas plumosos (Silva, 1975; Boldrini et al., 2005). O fruto é uma cariopse, a qual apresenta o pericarpo soldado em toda a sua extensão à testa da semente, deixando ver na base, do lado dorsal, o embrião superficial, e do lado ventral o hilo. Outra característica é apresentar endorsperma abundante (Boldrini et al., 2005).

A duração do ciclo vegetativo do arroz, isto é, o número de dias que decorre desde a emergência até a maturação, é muito variável segundo a variedade considerada e as condições de solo e de clima, podendo, no entanto fixar-se de 80 a 220 dias para as variedades da

subespécie *indica* e de 120 a 180 dias para as da subespécie *japonica* (Silva, 1975).

A nutrição mineral é um fator importante envolvido no crescimento e desenvolvimento vegetal e, portanto, na sua produtividade. Entre os elementos minerais essenciais, o Ferro (Fe) é um micronutriente de grande importância devido à sua implicação em processos fundamentais como fotossíntese, respiração, fixação de nitrogênio e síntese de DNA e às suas propriedades físico-químicas, participando em grande parte das reações redutivas básicas (Briat et al., 1995; Briat & Lobréaux, 1997). Além disso, o ferro tem papel essencial como componente de diferentes enzimas envolvidas na transferência de elétrons (reações redox) como citocromos e age como um co-fator de enzimas essenciais envolvidas na síntese de fithormônios (enzimas formadoras de etileno, por exemplo) (Bouzayen et al., 1991; Siedow, 1991). Cerca de 75% do ferro na folha está presente nos cloroplastos, como fitoferritina e ferredoxina, proteína que se sabe estar envolvida na transferência de elétrons no processo fotossíntese, sendo reversivelmente oxidado de Fe^{2+} a Fe^{3+} durante a transferência de elétrons. Portanto, a deficiência de ferro afeta em muito a fotossíntese (Taiz & Zeiger, 2004).

Em nível celular a alta reatividade desse metal pode vir a causar severos problemas. As mesmas propriedades físicas que permitem que o ferro funcione como um eficiente cofator e permita reações de catalisação em reações redox controladas permitem que o mesmo funcione como uma potente toxina quando não é protegido de biomoléculas suscetíveis. Várias reações intracelulares utilizam oxigênio molecular como aceptor de elétrons produzindo superóxido (O_2^-) ou peróxido de hidrogênio (H_2O_2). Essas espécies não são prejudiciais *per se*, mas contribuem para a geração de espécies reativas de oxigênio, no caso, o radical hidroxila ($^{\bullet}OH$). Sua formação é catalisada por ferro através da Reação de Fenton (Hell & Stephan, 2003):

$$Fe^{3+} + O_2^{\circ -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{-}OH + {}^{\bullet}OH$$

Resumida:

$$O_2^- + H_2O_2 \rightarrow O_2 + {}^{-}OH + {}^{\bullet}OH$$

Um dos mecanismos para a tolerância ao excesso de ferro em plantas pode ser a capacidade de tornar o ferro absorvido indisponível. O armazenamento de ferro pode ocorrer no espaço apoplástico, formado pelo *continuum* de paredes celulares de células adjacentes

bem como o espaço extracelular, em mitocôndrias (Zancani et al. 2004), plastídios (Seckback, 1982) e também no vacúolo, onde o baixo pH e altas concentrações de ácidos orgânicos representam condições ótimas para o depósito de ferro, dependendo do órgão vegetal e da espécie em questão (Briat & Lobréaux, 1998). O vacúolo é capaz de seqüestrar ferro, além de outros metais, tanto como um mecanismo de desintoxicação celular como de armazenamento, permitindo o crescimento celular quando em um ambiente com baixa disponibilidade de ferro (Santos & Costa de Oliveira, 2007). Diferentes proteínas presentes no tonoplasto são responsáveis pela translocação de metais através desta membrana. Em mitocôndrias e plastídios, a proteína ferritina, é utilizada como meio de armazenamento de ferro, sendo comum em plantas (Briat & Lobréaux, 1998).

A ferritina parece ser um componente importante para o controle da homeostase do ferro em eucariotos, pois constitui uma classe de proteínas armazenadoras de ferro amplamente distribuídas, que consiste em esferas formadas por 24 subunidades simetricamente relacionadas que formam uma cavidade oca, sendo capaz de armazenar até 4500 átomos de ferro por molécula em seu interior (Harrison & Arosio, 1996). Ferritinas são encontradas em diversos organismos, como animais, vegetais e bactérias (Briat et al., 2010).

Ferritinas vegetais têm sua sequência de aminoácidos altamente conservada com a de mamíferos (Andrews et al., 1992). Contudo, diferentemente de ferritinas encontradas em outros organismos, em plantas sua regulação se dá de maneira diferente. Enquanto em animais essa regulação é traducional (Arosio et al., 2008), em plantas se dá pelo controle transcricional (Lescure et al. 1991). A síntese de ferritina também é controlada pelo status de ferro no interior da célula, podendo ser acumulada no caso de excesso de ferro para que o metal seja armazenado de maneira atóxica para a célula, de forma que não reaja com oxigênio (Briat et al., 1995).

A ferritina parece estar intimamente relacionada com o desenvolvimento vegetal, contudo podendo exercer diferentes papéis. Em ervilhas, o ferro armazenado em ferritinas corresponde a 92% do total de ferro encontrado em embriões maduros (Marentes & Grusak, 1998), evidenciando o importante papel da ferritina na germinação. Contudo, a mesma parece estar envolvida com outros processos em sementes de Arabidopsis. Estima-se que não mais de 5% do ferro dessas sementes esteja armazenado em ferritinas (Ravet et al., 2009).

Arabidopsis thaliana possui quatro genes que codificam ferritina (Petit et al., 2001). Apesar de serem estruturalmente conservados, são expressos sob diferentes condições. Em

situações de excesso de ferro, *AtFer1* e *AtFer3* são majoritariamente expressos (Petit et al., 2001). *AtFer4* codifica uma ferritina mitocondrial em plantas tratadas com ferro (Tarantino et al., 2009) e *AtFer2* é expresso em sementes e sua expressão responsiva a ácido abscísico (Petit et al., 2001).

Dois genes que codificam para a proteína ferritina foram identificados no genoma de arroz, *OsFER1* e *OsFER2* (Gross et al., 2003). Em plantas expostas a tratamentos com cobre, Paraquat, nitroprussiato de sódio e excesso de ferro foi observado aumento na abundância de transcritos de ferritina, particularmente de *OsFER2* (Stein et al., 2009).

Dentre as famílias envolvidas na homeostase de metais, membros da família NRAMP (Natural-Resistance-Associated Macrophage Protein) foram descritos como tendo um papel importante na homeostase de ferro em diferentes organismos. Contudo, os membros desta família gênica possuem amplo espectro de especificidade no transporte de metais, incluindo ferro, manganês, cobalto, zinco, cobre, cádmio, níquel (Ňuňuková et al., 2010) e vanádio (Ueki et al., 2011). Alguns membros desta família foram caracterizados utilizando a técnica de expressão em oócitos de *Xenopus* (e.g. Agranoff et al., 2005, Ueki et al., 2011). Em *Arabidopsis* foram isolados seis genes homólogos à família NRAMP (Mäser et al., 2001), sendo que destes, *AtNRAMP3* e *AtNRAMP4* se mostraram capazes de complementar uma linhagem de levedura mutante, defectiva para a absorção de ferro (Thomine et al., 2000). Em estudo subseqüente, foi demonstrado que AtNRAMP3 é um transportador vacuolar (Thomine et al., 2003). Foi demonstrado também que estes transportadores são capazes de mediar transporte de ferro em *Arabidopsis*, estando envolvidos no efluxo de ferro dos vacúolos na semente durante a germinação (Lanquar et al., 2005).

Através de análises *in silico*, foi possível observar que, dentre as oito proteínas NRAMP previstas com base no genoma de arroz (Gross et al., 2003), o transportador OsNRAMP7 possui a maior homologia com as proteínas AtNRAMP3 e AtNRAMP4, 68% para ambas. Em um trabalho recente, nosso grupo observou correlação negativa significativa entre a expressão de *OsNRAMP7* em folhas bandeira durante o período de enchimento do grão e a concentração final de ferro e zinco em grãos (Sperotto et al., 2010). Esse resultado sugere o papel da proteína OsNRAMP7 no seqüestro de metais para o vacúolo em folhas, resultando em uma menor disponibilidade do metal a ser transportado para panículas. O papel do gene *OsNRAMP7* na alocação de metais para o grão também foi sugerido por sua co-localização com um QTL, capaz de explicar a variação fenotípica da concentração de ferro (Stangoulis et al., 2007) e zinco (Garcia-Oliveira et al., 2009) nos grãos em 14% e 13%,

respectivamente.

Em um trabalho pioneiro, Boorer et al. (1992) utilizaram a técnica de análise funcional de uma proteína de *Arabidopsis thaliana* em oócitos de *Xenopus*, definindo a afinidade da proteína transportadora ao substrato. Nessa técnica, cRNA referente ao gene em estudo é injetado em oócitos de *Xenopus laevis*, os quais possuem grande quantidade de enzimas, organelas e proteínas, produzindo assim a proteína de interesse. Uma das grandes vantagens do método é que há baixíssima atividade de transporte na membrana plasmática dessas células, o que garante background inexistente ou muito baixo da atividade de transporte para interferir com os resultados obtidos pela proteína de interesse, além de estudos de eletrofisiologia serem facilitados por se tratarem de células grandes (Miller & Zhou, 2000). Outra vantagem desta técnica se dá ao fato de a célula poder ser exposta a diferentes metais, ligados ou não a diferentes quelantes (Koike et al., 2004), permitindo investigar não somente a especificidade do transportador em relação ao metal, como também a forma química preferencial de transporte do metal através do transportador em estudo. Proteínas vegetais vacuolares foram expressas em oócitos e a direção do transporte *in planta* foi identificada avaliando a diferença entre o transporte do metal em questão entre plantas mutantes que não continham a proteína transportadora e plantas do tipo selvagem (Chopin et al., 2007).

Membros da família NRAMP já foram caracterizados utilizando esta técnica (Gunshin et al., 1997; Okubo et al., 2003; Agranoff et al., 2005; Ueki et al., 2011), contudo nenhum deles pertencente ao reino vegetal. Em um trabalho recente, realizado em colaboração com Dr. Anthony Miller (John Innes Centre, Reino Unido), foi possível observar que oócitos de *Xenopus* injetados com o mRNA de *OsNRAMP7* foram capazes de absorver ferro na forma Fe^{2+} (Capítulo 2 desta tese). Esta foi a primeira tentativa de caracterização de transporte desta proteína e os resultados obtidos sugerem não só sua viabilidade, como também o envolvimento da proteína no transporte de ferro e, consequentemente, na homeostase do metal em plantas de arroz.

Em *Arabidopsis*, a importância de vacúolos no armazenamento de ferro em sementes e sua remobilização durante a germinação foi documentada em nível molecular pela caracterização da atividade de transportadores vacuolares de efluxo, NRAMP3 and NRAMP4, e de influxo, VIT1. Foi então realizada a análise do gene de ferritina *AtFER2* em diferentes *backgrounds* genéticos que possuem a homeostase ferro dos compartimentos plastídicos e vacuolares afetados (mutantes *knockout fer*, *nramp* e *vit*, e plantas superexpressando as proteínas NRAMP e VIT) (Ravet et al., 2009). Tais estudos revelaram que a estabilidade da

ferritina em sementes depende da alocação apropriada do ferro do vacúolo para os plastídios, evidenciando uma possível comunicação entre os compartimentos de armazenamento vacuolares e plastidiais de ferro em sementes. Esses resultados indicam uma resposta integrada quanto à homeostase de ferro nas células.

A compreensão dos mecanismos de regulação da homeostase do ferro em plantas é de fundamental importância tanto do ponto de vista agronômico (possibilitando mais produtividade em plantas que não sofram os efeitos danosos da deficiência ou do excesso de ferro) quanto do ponto de vista da nutrição humana (possibilitando a produção de alimentos de origem vegetal com altos níveis de ferro disponíveis para absorção pelo sistema digestivo humano) (Gura, 1999; Grotz & Guerinot, 2002).

A caracterização de tais mecanismos em arroz é igualmente importante do ponto de vista científico, uma vez que o arroz desponta como planta modelo (fisiológico e genético) para as monocotiledôneas, fazendo par à dicotiledônea *Arabidopsis thaliana*. O arroz foi a segunda Angiosperma a ter o seu genoma completamente seqüenciado (Burr et al., 2005), sendo escolhido como organismo modelo para seqüenciamento entre as monocotiledôneas por sua importância agronômica, pequeno tamanho do genoma (392 Mpb – o menor das gramíneas) e sua relação de sintenia com outras espécies de cereais (IRGSP, 2005). O arroz tem sido extensamente manipulado geneticamente, uma vez que é visto como modelo de pesquisa para outras culturas (Devos & Gale, 2000). Dessa forma, permite que seja utilizado para a realização de estudos de colinearidade molecular em outras espécies de gramíneas, e assim, com base na sintenia, identificar e caracterizar genes de interesse em espécies relacionadas.

Além disso, como alimento básico de grande parte da população humana (e indiscutivelmente da brasileira) o arroz foi escolhido para experimentos pioneiros de fortificação alimentar através da engenharia genética, já tendo sido obtidas plantas transgênicas com maiores teores de betacaroteno (precursor da vitamina A), uma vez que a deficiência de vitamina A também é um problema grave de saúde pública em países não desenvolvidos (Ye et al., 2000; Paine et al., 2005).

Visto que a homeostase de ferro parece resultar de uma resposta integrada na célula, é necessário que o estudo abranja não apenas uma das formas de armazenamento de ferro nas células, dado que ambos, ferritina e vacúolo, se mostraram intimamente ligados em estudos anteriores (Ravet et al., 2009).

Justificativa

Estudos acerca do desenvolvimento vegetal destacam o papel da ferritina como um reservatório de ferro transiente para importantes processos ferro-dependentes como fotossíntese e fixação de nitrogênio. Trabalhos recentes (Silveira et al., 2009, Stein et al., 2009) propõem o envolvimento da ferritina na proteção ao estresse oxidativo em cultivares de arroz submetidas a tratamento de excesso de ferro. Plantas de arroz são expostas frequentemente a excesso de ferro, devido às condições resultantes do cultivo em condições de alagamento. A produção de ferritina pode também ser induzida por outros fatores, visto que a luz pode vir a induzir a produção da proteína (Stein et al., 2009). Contudo, mais trabalhos são necessários para determinar o papel da ferritina na resposta ao estresse oxidativo. Estudos utilizando mutantes são ferramentas úteis para a identificação ou a confirmação da função de um gene. A utilização de uma linhagem de arroz mutante para *OsFer2* no presente trabalho tem como intuito gerar dados que contribuam para a compreensão do papel da proteína ferritina na homeostase de ferro em plantas de arroz.

O arroz é um dos principais alimentos da população humana, mas contém baixas concentrações de minerais essenciais, como o ferro, nos grãos. Como a principal deficiência mineral em humanos é a de ferro, vários esforços tem sido feitos visando compreender e manipular os mecanismos responsáveis pela alocação de ferro para os grãos de arroz. A proteína OsNRAMP7 foi identificada dentre as proteínas co-localizadas com um Quantitative Trait Loci (QTL) que explica parte da variação fenotípica da concentração de ferro (Stangoulis et al., 2007) e zinco (Garcia-Oliveira et al., 2009) nos grãos de arroz. Além disso, foi observada correlação negativa significativa entre a expressão de *OsNRAMP7* em folhas bandeira durante o período de enchimento do grão e a concentração final de ferro e zinco em grãos (Sperotto et al., 2010), em trabalho realizado no Laboratório de Fisiologia Vegetal da UFRGS. Por meio de análises *in silico*, foi constatado que OsNRAMP7 tem identidade de 68% com as proteínas AtNRAMP3 e AtNRAMP4 de *Arabidopsis thaliana*. Foi demonstrado que AtNRAMP3 e AtNRAMP4 são proteínas transportadoras de ferro em vacúolos (Lanquar et al., 2005). Até o momento, pouco se sabe sobre o transporte intracelular de ferro em arroz. Determinar se OsNRAMP7 é, de fato, um transportador de ferro, poderá aumentar a compreensão dos mecanismos necessários para a manutenção da homeostase deste metal em

plantas de arroz.

Desta forma justifica-se o presente trabalho, pelo qual pretende-se contribuir para o aprofundamento, expansão e difusão das pesquisas sobre a homeostase de ferro em plantas de arroz. Os resultados obtidos neste trabalho também poderão ser úteis para o desenvolvimento de estratégias visando aumentar os teores de ferro no grão de arroz e a tolerância de plantas de arroz ao excesso de ferro.

Objetivos

Geral

Pelo presente trabalho tem-se como objetivo principal contribuir para a elucidação de mecanismos de regulação da homeostase de ferro em plantas de arroz (*Oryza sativa* L.). Para tanto, o papel da proteína de armazenamento ferritina será investigado em relação aos mecanismos de defesa e proteção contra o estresse oxidativo gerado pelo excesso de ferro. Da mesma forma, determinar se a proteína transportadora OsNRAMP7 está envolvida com o transporte de ferro em plantas de arroz pode esclarecer aspectos importantes da homeostase deste metal.

Específicos

1. Analisar a presença dos transcritos de *OsFer1* e *OsFer2*, bem como da proteína ferritina, em plantas mutantes de arroz contendo inserção do transposon Tos17 no gene *OsFer2* (linhagem NG0250) e plantas do tipo selvagem (WT).
2. Investigar a propriedade protetora da proteína ferritina frente ao estresse oxidativo gerado pelo excesso de ferro livre intracelular.
3. Expressar o mRNA de *OsNRAMP7* em oócitos de *Xenopus laevis* isolados, caracterizando a capacidade da proteína transportar ferro como potencial substrato.
4. Analisar características estruturais da proteína OsNRAMP7 e motivos relacionados ao transporte de metais.

Capítulo 1

Artigo a ser submetido como revisão a periódico indexado

Review

Iron homeostasis in Plants: current knowledge on mechanisms and genes.

1. Introduction

Mineral nutrition is an important factor involved in plant development. Among the essential mineral elements, iron is a micronutrient of great importance due to its physical-chemical properties, participating in most of the basic reductive reactions. It is also essential for basic processes as photosynthesis, respiration, nitrogen fixation and DNA synthesis (Briat et al., 1995; Briat & Lobréaux, 1997). Iron has an essential role as component of different enzymes involved in electron transfer (redox reactions) such as cytochromes and acting as a co-factor of essential enzymes involved in phytohormone synthesis (eg ethylene synthesis) (Bouzayen et al., 1991; Siedow, 1991). About 75% of the iron in leaves is in chloroplasts, as phytoferritin and ferredoxin, proteins involved in electron transfer in photosynthesis, reversibly oxidized from Fe^{2+} to Fe^{3+} during electron transfer. Therefore, iron deficiency deeply affects photosynthesis (Msilini et al., 2011).

Owing to such important characteristics, we aimed to review the current knowledge on iron uptake mechanisms, trafficking and storage in plants.

2. Iron uptake

Despite the fact that iron is the second most abundant metal in soils (first being aluminum), it may not be available or easily absorbed by plants. For that reason, plants have developed two strategies to assure iron absorption when exposed to iron deficiency conditions. Two mechanisms underlying iron deficiency responses have been characterized and in this situation iron is absorbed either by chelation or reduction strategies.

2.1 Reduction strategy

Iron absorption in non-grass plants is directly related to the root capacity to reduce iron from Fe^{3+} to Fe^{2+} (Figure 1a). Also called Strategy I, it relies on the capacity of H^+-ATPases, located in the epidermis, to release protons to the rhizosphere (Santi et al., 2005). Increment of H^+ in the soil lowers the pH, increasing iron solubility. To be transported into the cells, iron must be reduced from Fe^{3+} to Fe^{2+}. In Arabidopsis, this is carried out by a

NADPH-dependent ferric chelate reductase, AtFRO2 (Robinson et al., 1999). This reduction is crucial for iron absorption in non-grass plants. Heterologous expression of *AtFRO2* in Soybean (*Glycine max* Merr.) led to an increase in Fe^{+3} reduction in both roots and leaves. This enhanced activity reduced chlorotic phenotype and increased chlorophyll concentration (Vasconcelos et al., 2006).

It is only after this step that roots can absorb iron. AtIRT1 is a divalent metal transporter, with affinity not only for iron (Eide et al., 1996), but to Zn, Mn, Co, Cd (Korshunova et al., 1999) and Ni (Schaff et al., 2006) as well. Mutant *irt1* plants present increased photosensitivity and altered chlorophyll fluorescence parameters. Plants are also chlorotic and growth and fertility are significantly reduced, symptoms related to impaired iron transport (Varotto et al., 2002). These evidences suggest a primary role of IRT in iron absorption under iron-deficiency conditions.

2.2 Chelation strategy

Grasses make use of a different mechanism to absorb iron, called Strategy II. This process, based on chelation of the iron molecule, occurs when grass roots release phytosiderophores (PS) into the rhizosphere (Figure 1b). Of the PS molecules, maize (*Zea mays* L) and rice (*Oryza sativa* L.) secrete 2'-deoxymugineic acid (DMA). However, other grass species secrete hydroxylate DMA, releasing different mugineic acids (MAs) into the soil. DMA is a molecule able of chelating Fe^{3+}, while transporters of the YSL family transport this complex through the root (Ueno et al., 2007).

The first Fe-PS transporter identified was YS1 in maize. Mutant *ys1* plants are deficient in Fe^{3+}-MA uptake, leading to a constitutive iron-deficiency response. Due to the lack of iron, leaves display interveinal chlorosis (Curie et al., 2001). In rice, *OsYSL15* is expressed in root epidermis and stele, being induced by iron-deficiency (Inoue et al., 2009). It has also been determined that this protein has high affinity for Fe^{3+}-DMA (Inoue et al., 2009). Rice *osysl15* mutant plants showed chlorotic phenotype under iron deficiency conditions and reduced iron concentration in all organs (Lee et al., 2009).

Since this strategy is not based on iron solubility in the rhizosphere, it is less sensitive to pH in the soil. The capacity of plants to release PS and the availability of iron in the environment are the limiting factors for this strategy.

2.3 Combined strategy

A combination of the strategies described above was also reported. Rice presents not only the PS release to the rhizosphere, but also to reduce Fe^{3+} to Fe^{2+} and to transport the latter using the OsIRT1 transporter (Ishimaru et al., 2006) (Figure 1c). When Fe^{3+}-DMA and Fe^{2+} were supplied to rice plants, a Positron-Emitting Tracer Imaging System (PETIS) experiment allowed to observe that both forms were absorbed. When *OsIRT1* was over-expressed in rice plants, iron and zinc content was elevated in shoots, roots and mature seeds (Lee & An, 2009).

Rice is less tolerant to calcareous soils then barley or maize, since it releases less PS into the rhizosphere than these species (Nagasaka et al., 2009). Compensating this deficit with the expression of a divalent metal transporter, such as OsIRT1, may enable rice to sustain normal growth under iron-limiting conditions.

3. Translocation

A sequence of processes that involve several metal chelators and transporters are required for the safe translocation of iron within the plant. Chelators are used by plants due to the metal's chemical properties, such as high reactivity and poor solubility. It aims to prevent formation of reactive oxygen species (ROS) and hydroxyl radical ($^{\bullet}OH$), a formation catalyzed by iron through the Fenton Reaction and a possible precipitation of the metal (Hell & Stephan 2003).

3.1 Citrate

Fe^{3+}-citrate is known to be the major form of iron present in xylem exudates (Grotz & Guerinot 2006) and is involved in long-distance transport of iron (Yokosho et al., 2009). FRD3, a multidrug and toxic compound extrusion (MATE) transporter, is a gene mainly expressed in roots and is involved with the translocation of iron-citrate chelates (Durrett et al., 2007). Due to its localization in the plasma membrane of cells in the pericicle and vasculature (Green & Rogers, 2004), FRD3 seems to be responsible for the iron-citrate transport to the xylem (Durrett et al., 2007).

The *Arabidopsis thaliana ferric reductase defective3* (*frd3*) mutant exhibits a chlorotic phenotype and constitutive expression of iron uptake responses. As a result, the mutant accumulates iron in roots, however not being able to translocate the metal via the xylem to aerial parts, leading to iron deficiency in leaves and a chlorotic phenotype. Of the six

orthologs found in rice, *OsFRDL1* is also expressed in the pericicle of root cells and is found to transport citrate when expressed in *Xenopus* oocytes (Yokosho et al., 2009).

Also, the *osfrdl1* loss of function insertion mutant has a similar phenotype to *frd3* – leaf chlorosis, lower leaf iron concentration and precipitation of iron in the root. Although there was a decrease in the Fe^{3+} concentration in xylem sap, the same was not observed for Fe^{2+}, suggesting the use of another chelating molecule by the plant to transport Fe^{2+} through the xylem (Yokosho et al., 2009).

3.2 Nicotianamine

NA is a non-proteogenic amino acid ubiquitous in plants that chelates both Fe^{2+} and Fe^{3+}, in addition to other divalent metals (Haydon et al., 2007). The chelation properties of NA, such as affinity and stability, are the highest at neutral and mild basic pHs, making the molecule more suitable for phloem transport than other compounds, such as organic acids (Curie et al., 2009).

Most of the information regarding NA has come from the tomato *chloronerva* (*chl*) knock-out mutant, defective in NA synthase. Plants lacking NA show interveinal chlorosis in young growing leaves and constitutively activate their root iron-uptake systems, despite their mature leaves containing a high amount of iron (Conte & Walker, 2011). As a result of the apparently immobility of iron in the phloem, younger leaves lack iron. This contrasts with the total iron transported to older leaves, supposedly via xylem, which remains normal (Conte & Walker, 2011).

Both *Arabidopsis* and rice have proteins described as being involved in transporting Fe-NA complexes. Of the eight members in *Arabidopsis*, YSL1 and YSL2 have been described as transporters of Fe-NA (DiDonato et al., 2004). Out of the 18 members of the YS Like family in rice, OsYSL2 has been demonstrated to transport Fe-NA complexes through heterologous expression in *Xenopus* oocytes (Koike et al., 2004), but not Fe^{3+}-PS.

3.3 2'-deoxymugineic acid (DMA)

DMA is a PS responsible for chelating Fe^{3+} molecules in the rhizosphere to enable its absorption by YSL family members located in roots of graminaceous plants (Inoue et al., 2009). DMA may also be involved in iron translocation within the plant. It was detected in the phloem sap of rice leaves (Mori et al., 1991; Higuch et al., 2001) and *OsNAAT1* (Inoue et al., 2009), *OsNAS1–3* (Inoue et al., 2003) and *OsDMAS1* (Bashir et al., 2006) are expressed in the

phloem companion cells of iron deficient leaves. These genes encode key enzymes in the biosynthetic pathway of MAs.

3.4 ITP

Another iron-binding protein was identified when analyzing the phloem-mediated transport of micronutrients during the germination of *Ricinus communis* (Kruger et al., 2002). The Iron Transport Protein (ITP) was identified in phloem exudates, where it appears associated to the radio labeled iron supplied to the plantlets. The protein showed high affinity to Fe^{3+} but not to Fe^{2+} *in vitro*, where it also complexes Cu^{2+}, Zn^{2+} and Mn^{2+} (Kruger et al., 2002).

The ITP from castor bean shows high similarity to the stress-related family of late embryogenesis abundant (LEA) proteins. The most similar annotated sequences in both *Arabidospsis* and rice are related to stress induced responses. The *Arabidopsis* sequences are apparently involved in responding to water stress, while rice's are to both to water and saline stress (Kruger et al., 2002).

3.5 Intercellular

Once absorbed by roots, iron is likely complexed by chelating molecules (Figure 2) due to the metal's chemical properties. It is then translocated to the xylem as part of the distribution process within the plant. An important molecule for xylem movement of iron is citrate. Iron transport into xylem cells can be made by transporters such as AtFPN1 (Morrisey et al., 2009) or by proteins known to transport citrate, such as AtFRD3 (Durret et al., 2007) and OsFRDL1 (Yokosho et al., 2009).

Mechanisms involved in iron loading from xylem to phloem vessels have already been described. Is has been reported that OsIRT1 is involved in iron transport in the stele and that it is specific for Fe^{2+} (Ishimaru et al., 2006).

Members of the OligoPeptide Transporter (OPT) family were also described in iron translocation in plants, including genes of the YSL subfamily. Among 18 putative YSL genes identified in the rice genome, *OsYSL2* had its expression observed in phloem cells of the vascular bundles of leaves and leaf sheaths. It has been recently demonstrated that the protein encoded by this gene is vital for the long-distance transport of not only Fe, but also Mn. The RNAi (OsYSL2i) line increased iron concentration in roots while decreasing Fe and Mn concentrations in shoots. When the gene expression was driven by the sucrose transporter

promoter, iron concentration in the polished grain was increased by 4.4 fold (Ishimaru et al., 2010). These results indicate the importance of the OsYSL2 protein in Fe-NA translocation in rice plants.

Arabidopsis has eight members of the YSL gene family identified in its genome. Of them, YSL1, YSL2 and YSL3 have been characterized as important for metal homeostasis. Although it is thought that AtYSL2 is involved in Fe transport, conflicting results were obtained when groups attempted to establish its function. While DiDonato et al. (2004) reported restored growth of *fet3fet4* yeast mutant complemented with *OsYSL2* only when supplied with Fe-NA, Schaaf et al. (2005) claim that the observation could not be made in their experiment. However, additional experiments done by the latter group support the protein's involvement in iron homeostasis.

The Arabidopsis *ysl1ysl3* double mutant exhibited Fe deficiency symptoms, such as interveinal chlorosis, low concentration of iron in leaves and impaired mobilization of metals from leaves during senescence (Waters et al., 2006). Although the *ysl1ysl3* mutant showed interveinal chlorosis, as the *chl* mutant, its Fe deficiency response remained unaltered. This could be an indication of a tissue-specific response in *Arabidopsis.* These proteins are also involved in translocation of Fe into seeds, since the concentrations of Fe, Zn and Cu were lower when the proteins were impaired. Seed fertility was also reduced in the double mutant, since anthers and embryos had defective development (Waters et al., 2006).

Of the same family, AtOPT3 has an essential role in embryo development. A mutation on this gene induced continued Fe deficiency responses in roots, high level of Fe in tissues due to continuous absorption, and development of necrotic areas. Despite the high amount of Fe in plants, *atopt3* mutants showed less Fe in seeds, indicating an important role for the protein in Fe translocation to developing seeds (Stacey et al., 2008). Among the 15 putative members of the OPT family in *Oryza sativa* cv. japonica (Gomolplitinant & Saier, 2011), the one with highest identity to AtOPT3 is Osa13 (GenBank accession number 115455379). Further studies are required to determine if this rice putative protein has indeed functions related to its Arabidopsis homologue.

3.6 Intracellular

Once inside the cell, iron must be compartmentilized, to avoid toxic effects possibly generated by oxidative stress. Iron storage compartments and molecules are of great importance for supplying iron to essential processes, maintaining cell functions. An overview

of mechanisms involved in compartimentalization are described in Figure 3.

3.6.1 Vacuole

The largest iron storage compartment, the vacuole, is essential for maintaing iron homeostasis. It is of special importance in seeds, where it provides iron before the organism is capable of aquiring the metal from the environment (Lanquar et al., 2005, Kim et al., 2006). Two proteins, AtFPN2 and AtVIT1, are known to be involved in iron loading to the vacuole. *AtFPN2* has its expression localized at the outermost layers of *Arabidopsis* roots (Morrisey et al., 2009). It also appears to be involved in translocation of other metals besides iron (Schaaf et al., 2006). This is coherent with the fact that *fpn2* mutants are more sensitive to Co and Ni than the wild type (WT) (Morrisey et al., 2009).

AtVIT1 influx protein is involved in iron loading to seeds and is highly expressed during germination and development of young seedlings (Kim et al., 2006). Heterologous expression of *AtVIT1* was able to complement the iron-sensitive phenotype of the yeast mutant line *ccc1*. Upon expression, an increase in vacuolar iron content was observed, confirming the protein role as a vacuolar iron transporter (Kim et al., 2006).

In Arabidopsis seeds, iron is mainly located in the provascular strands of the developing embryo. However, when the AtVIT1 protein had its function impaired this was no longer observed. Although mutant seeds had the same content of iron as WT, its seeds didn't have the same germination development as WT seeds in alkaline pH conditions (Kim et al., 2006).

The same was observed in mutants of the vacuolar transporters AtNRAMP3 and AtNRAMP4. Arabidopsis *nramp3 nramp4* double mutants have impaired germination in low iron conditions, despite having the same seed iron content as WT. This happens due to seed incapacity of mobilizing iron from vacuoles. Analysis using Energy Dispersive X ray (EDX) showed that, after two days of germination, the vacuole of mutant seedlings still contained iron, while WT had remobilized the iron for a proper germination. This is especially important due to the fact that *AtIRT1*, an iron uptake transporter, is expressed only after the third day of germination (Lanquar et al., 2005).

Our group identified eight NRAMP family members in the rice genome (Gross et al., 2003). In a recent work, a significant negative correlation was found between OsNRAMP7 (LOC_Os12g39180) expression in flag leaves during grain filling and final Fe and Zn concentrations in the grain (Sperotto et al., 2010). To investigate OsNRAMP7 influence on

iron homeostasis, heterologous expression of the protein was conducted in *Xenopus* oocytes. We observed that oocytes expressing the protein presented a significant increase in iron content, in two iron concentrations tested (Santos et al., data not published). This demonstrates that OsNRAMP7 is able to perform transmembrane iron transport and is, indeed, involved in cellular iron homeostasis.

3.6.2 Chloroplast

The vacuole is the major iron storage in seeds. In leaves, however, plastids are responsible for up to 80% of the iron content (Shikanai et al., 2003). Iron concentration must be tightly regulated in chloroplasts, since the photosynthetic electron transport chain produces ROS, which may react with iron leading to oxidative damage. Although not much is known about iron trafficking in this organelle, recent findings have shed light on the transport mechanism involved.

To be transported into chloroplasts, Fe must first be reduced from Fe^{3+} to Fe^{2+}. *AtFRO7*, a member of the ferric reductase oxidase family, is localized in chloroplasts. Chloroplasts of Arabidopsis *fro7* mutants had 33% less iron than WT, and defects in photosynthetic transport were also observed (Jeong et al., 2008). This could be an indication of the existence of an influx iron transporter specific for divalent metals.

Recent findings in sugar beet (*Beta vulgaris* L.) have demonstrated that uptake transporters present in intact chloroplasts have a preference for ferric iron complexes as substrate. It also supports the existence of an active ferric chelate reductase, localized in the inner membrane and that uses NADPH. Once inside the inner envelop, these ions are incorporated into Fe-S/heme cofactors (Solti et al., 2012).

In a screen for metal transporters in plastids, *Arabidopsis'* Permease In Chloroplasts1 (PIC1) was identified. Arabidopsis *PIC1* mutants had chloroplast development impaired and an increase in ferritin clusters. They also presented dwarfism and chlorotic phenotype (Duy et al., 2007). Yeast complementation assay confirmed that AtPIC1 transports iron (Duy et al., 2007), although it is not certain if Fe^{3+} or Fe^{2+}. PIC1ox (PIC1-overexpressing lines), however, resembled ferritin knock-out plants. These plants presented symptoms as oxidative stress and leaf chlorosis, which could be due to an increase of iron concentration in chloroplasts. PIC1 function in iron homeostasis could also be indicated by the results obtained when it was overexpressed, leading to impaired plant growth, especially in fruit development (Duy et al., 2011).

3.6.3 Mitochondria

As chloroplasts, mitochondria have a large demand for iron, used as a cofactor in the respiratory electron transfer chain (Balk & Pilon, 2011). An iron influx transporter has not been characterized, but it is proposed that AtSTA1 and AtSTA2 are involved in Fe-S cluster efflux from the organelle. AtSTA1 belongs to a subfamily of *Arabidopsis* half-ABC transporters, is localized in the inner membrane and its ABC domains face the mitochondrial matrix. Chlorotic phenotype and stunted growth tendency were observed in *sta1* mutants (Kushnir et al., 2001). A defect in the maturation of Fe-S proteins seems to be related to this phenotype (Kispal et al., 1999).

Proteome analysis of mitochondria revealed the presence of a ferric chelate reductase AtFRO8 (Heazlewood et al., 2004). This could be an indication that the reduction strategy observed in chloroplast is also present in mitochondria.

4. Storage

Iron is an essential metal, required for several metabolic processes both in chloroplast and mitochondria. Iron is also stored in these organeles, as well as in the vacuole. As part of iron homeostasis in the whole plant, iron levels and compartmentalization are tightly regulated. Sequestration and chelation strategies are used by plants to prevent suffering from toxic effects of iron.

Once loaded to the vacuoles, molecules containing iron are stored complexed to globoids. The presence of globoids was directly linked to levels of iron in vacuoles (Lanquar et al., 2005). Both NA and phytates (PA) are molecules that can complex iron in globoids. If indeed iron is transported to or from the vacuole complexed with NA, members of the YSL family could be involved in the transport. In a proteomic analysis of isolated vacuoles of *A. thaliana*, AtYSL4 and AtYSL6 were identified as being present in the tonoplast (Jaquinod et al., 2007). Iron-phytate globoids are common in vacuoles due to a high binding capacity between PA-O-Fe. Each molecule of PA is capable of binding 2 to 4 Fe^{3+} ions (Bohn et al., 2008).

Different functions have been assigned to ferritin, depending on the species analyzed. In peas, ferritin seems to be the most important iron storage site in seeds, where it releases and provides the metal to the iron-containing proteins after germination (Becker et al., 1998).

However, no more than 5% of the iron present in *Arabidopsis* seeds is estimated to be stored in ferritins (Ravet et al., 2009). It would seem that in *Arabidopsis* the protein's most significant feature would be related to oxidative stress prevention.

Ferritins are found in chloroplasts, mitochondria and cell walls (Becker et al., 1998). They are composed of 24 subunits that may allocate from 2,000 to 4,500 Fe^{3+} atoms per protein. Iron is oxidized by the ferroxidase centre of the protein before being stored as Fe^{3+} inside the mineral core. Not only the protein prevents iron from reacting with compounds that might generate ROS, it actually consumes oxygen and hydrogen peroxide during the oxidation reaction (Arosio et al., 2009). Ferritins provide bio-available iron inside the cell and have yet some potential detoxification properties.

Unlike ferritin, frataxin (Fh) is found solely in mitochondria. Since it is strongly conserved, it is proposed that it should have similar roles in different organisms (Ramirez et al., 2011). Putative functions for this protein include assisting in Fe-S cluster assembly (Chen et al., 2002) and involvement in energy conversion and oxidative phosphorylation (Ristow et al., 2000). The protein appears to have an essential role in seed development, as it was observed that Arabidopsis knockout mutants (*atfh-2* and *atfh-3*) have an embryo lethal phenotype. A protective role against oxidative damage was proposed for frataxin as the mutants showed increased content of ROS and higher levels of transcripts of proteins known to be involved in oxidative stress responses (Busi et al., 2006). The frataxin from the monocots *Triticum aestivum*, *Oryza sativa* and *Zea mays* present 77%, 76% and 75% similarity with AtFH, respectively (Busi et al., 2004).

5. Transcription factors related to iron homeostasis in rice

Two transcription factors were shown to have influence either in sensing cellular iron status or iron accumulation in rice plants. IDEF1 has a particular characteristic that allows it to sense iron status and, therefore, positively regulate most genes involved in iron uptake or utilization. It has His-Asp repeats and Pro-rich regions, known to bind Fe^{2+}. When these metal-binding regions are deleted from IDEF1, the plant fails to have a normal response to iron-deficiency and early iron-deficiency genes regulated by the transcription factor are not activated (Kobayashi et al., 2012).

OsARF12, also a transcription factor, has an influence in iron homeostasis in rice (Qi et al., 2012). Knockout plants showed lower concentrations of iron in leaves, roots and seeds

when compared to WT plants. It was also observed in these plants an alteration in the abundance of *mitochondrial iron-regulated* (*OsMIR*), *iron-regulated transporter* (*OsIRT*) and *short postembryonic root1* (*OsSPR1*) transcripts. *OsMIR*, which encodes a mitochondrial protein involved in iron homeostasis, has its transcription increased both in roots and shoots under iron deficiency conditions. In the lack of OsARF12, *OsMIR* was up-regulated and *OsSPR1* down-regulated. *OsIRT1* and *OsIRT2* transcripts were lower in roots when compared to WT. However, transcript levels were increased in leaves. Despite having different spatial expression, both roots and shoots showed lower iron concentrations than WT (Ishimaru et al., 2009).

Unlike the transcription factors described above, *OsWRKY80* is induced by iron excess instead of iron deficiency conditions (Ricachenevsky et al., 2010). The increase of transcripts is found throughout the plant, indicating a systemic response to the stress. *OsWRKY80* also responds to other stresses, such as drought and dark-induced senescence. This particular transcription factor is a member of the WRKY family, which is mostly, but not exclusively, found in plants. They are related to several processes such as senescence, plant defense and response to abiotic stresses (for review, see Rushton et al., 2012).

6. Biofortification

It has been previously described that rice possesses a combination of both iron absorption strategies. This enables rice to adapt more easily to diverse iron-limiting conditions. Molecules that are essential for iron chelation and transport within the plant have been described in rice as well. Among them, NA has been demonstrated to be of great value concerning rice biofortification. The overexpression of a single NAS gene, *OsNAS2*, increased iron concentration in rice grains by four-fold (Johnson et al., 2011). This appears to be the most successful attempt to increase iron and zinc concentrations in rice grains so far.

7. Conclusion and prospects

Due to conjunct and continuous efforts of several teams worldwide, we are now unraveling the processes regarding iron homeostasis in plants. Much progress was made toward understanding physiological and molecular mechanisms underlying the phenomena. A better understanding on release of PS to the rhizosphere will allow a complete

characterization of the iron absorption Strategy II, used by many economically important crops. Also, research is still needed to understand several mechanisms vital for intracellular iron transport, where knowledge is still at surface. It is known that chloroplasts and mitochondria have particular iron requirements. However, little is known about Fe intake by these organelles, how they interact regarding iron homeostasis and how the metal status is sensed and the signal distributed to maintain adequate levels within the cell.

Clarifying ferritin and vacuole functions both in vegetative tissues as in seeds provided important information about iron detoxification and storage. Findings in *Arabidopsis* were crucial for understanding the vacuole role as primary iron source in the seeds, enabling germination. Revealing aspects of signaling in the plant, how it adjusts metal uptake to the current condition, translocates it throughout the plant and then proceeds to storage will help fully understand iron homeostasis in plants. Disclosing this information will be of great value for agriculture and human nutrition.

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Figures

Figure 1

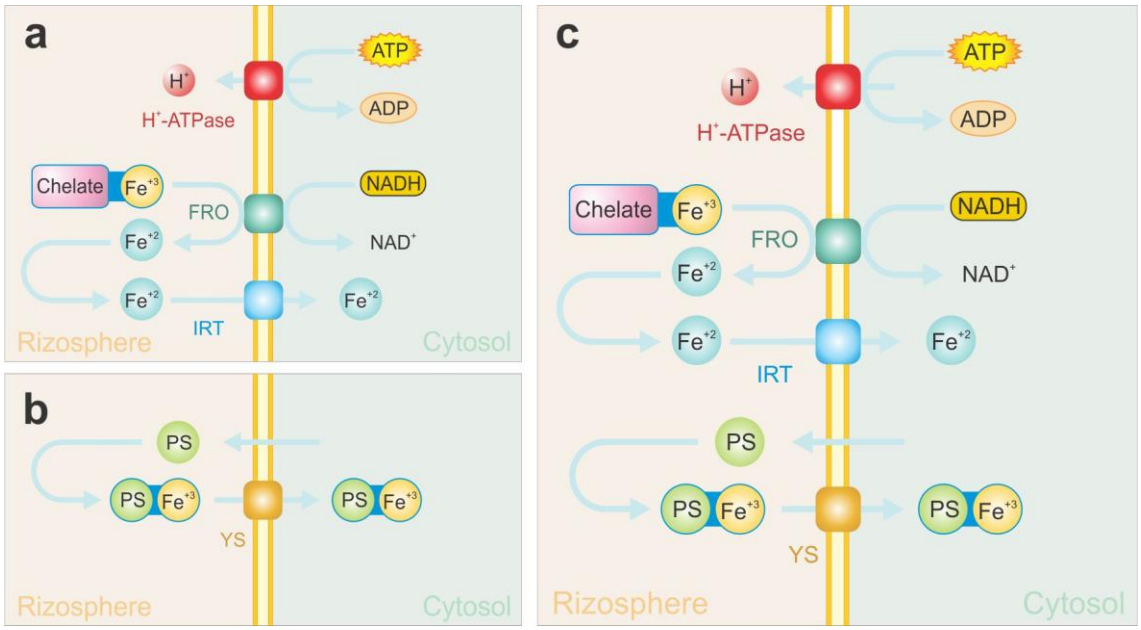

Figure 1: Strategies by which plants absorb iron from soil in iron deficiency conditions. **a.** Reduction strategy used by non-graminaceous plants. Protons are expelled by the H^+-ATPase. FRO, a ferric chelate reductase, is responsible for reducing Fe^{3+} to Fe^{2+} which is then transported inside the plant by the divalent metal transporter IRT. **b.** Chelation strategy, used by grasses, where phytosiderophores (PSs) are secreted by roots. These molecules easily chelate Fe^{3+} present in the soil, and the complexed molecule can be transported by Yellow Stripe (YS) proteins into the root. **c.** Combined strategy present in rice. Both acidification system and chelation by a PS are present in rice roots in iron limited environments.

Figure 2

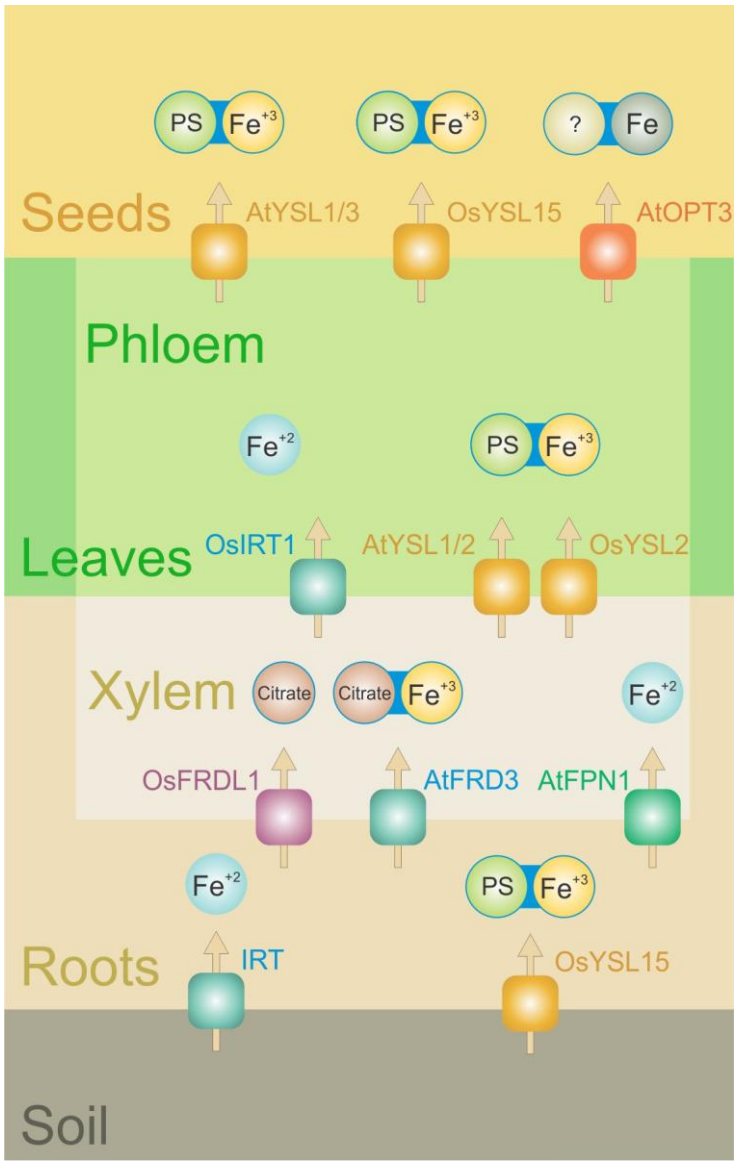

Figure 2: Schematic representation of iron chelation and long-distance transport in plants. Once absorbed by epidermal cells into the root, the metal must enter the vascular bundle. Proteins such as AtFPN1, AtFRD3 and OsFRDL1 are known to be involved with iron and/or citrate transport to the xylem. IRT family members were identified as iron transporters to the phloem. Also, members of the YS family transport Fe-PS into the phloem in both *Arabidopsis* and rice. NA appears to be the most important PS for long-distance transport of iron to seeds. Seeds of Arabidopsis mutants *ysl1ysl3*, *opt3* and *osysl15* present lower iron content, indicating the proteins are involved with iron loading to seeds.

Figure 3

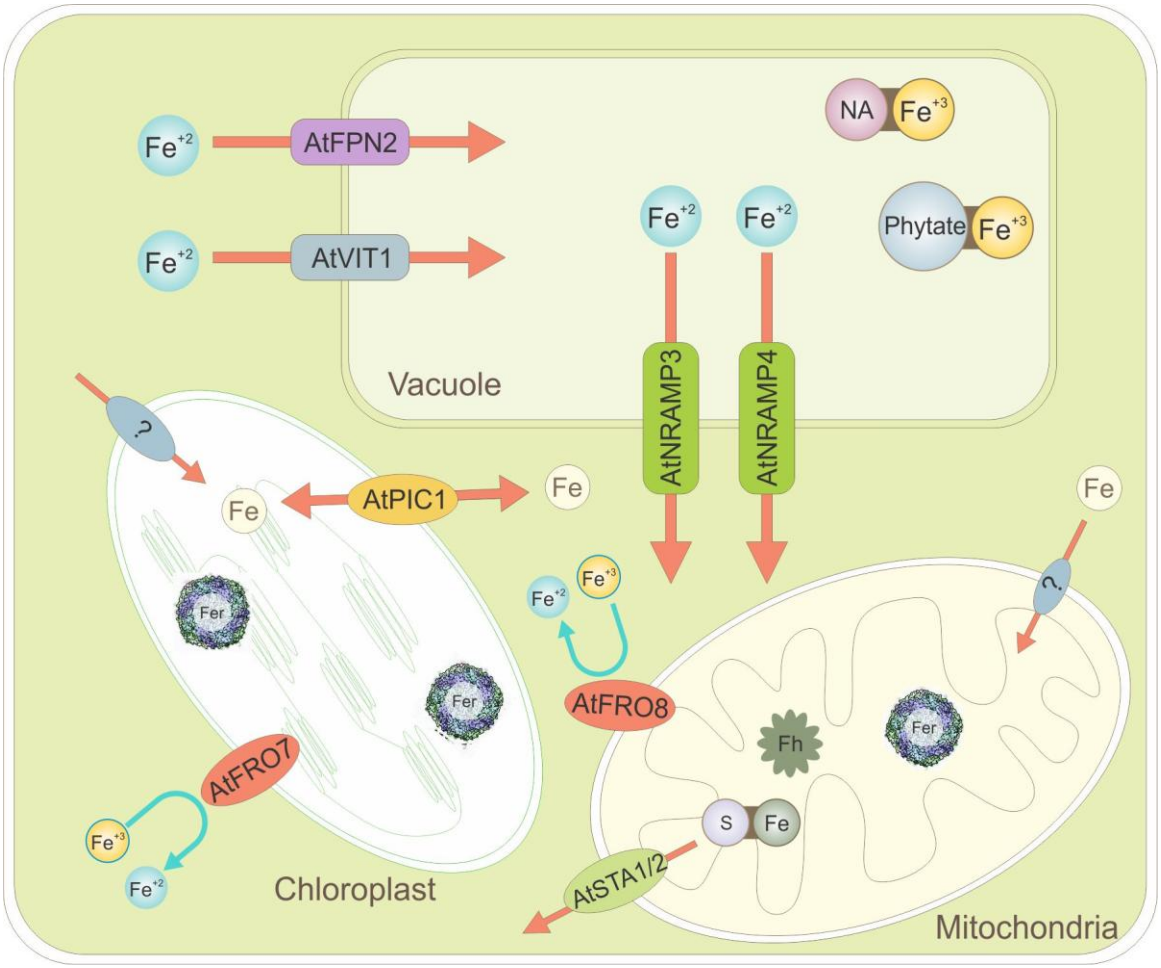

Figure 3: Schematic representation of iron transport and storage within the cell. In different tissues, iron is loaded to the vacuole by two transporters, AtFPN2 and AtVIT1. NA and phytate are known to chelate iron inside the vacuole. Iron remobilization from the vacuole is made by AtNRAMP3 and AtNRAMP4. The mechanism of iron transport to the chloroplast is not yet characterized, although it is known that Fe^{3+} must be reduced to Fe^{2+} by AtFRO7. It is possible that AtPIC1 (localized in the inner membrane) might play a role in iron loading. However, there is reason to believe that other proteins are involved in this task. Mechanisms involving ion loading in mitochondria have not been yet characterized, although it is known that AtSTA1 and AtSTA2 are involved in the metal efflux. Also, AtFRO8 was observed in proteomic analysis. Ferritin is an iron storage protein present both in mitochondria as chloroplasts. Frataxin is an iron sequestration protein present in mitochondria.

Capítulo 2

Iron Transport by the Rice OsNRAMP7 Protein

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Iron Transport by the Rice OsNRAMP7 Protein

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Abstract

Proteins from the NRAMP family from diverse organisms are known to transport a wide range of metals, playing important roles in cellular metal homeostasis. Some members of the family have been described as particularly participating in plant iron homeostasis. Of special interest in rice plants, the OsNRAMP7 protein was investigated in this work. Characteristic features, motifs DPGN and MPH, said to be involved in metal transport, were identified in the protein structure. To investigate the involvement of the rice OsNRAMP7 protein in iron transport, a complete version of the protein as well as a truncated form missing the first 15 amino acids, were expressed in *Xenopus* oocytes. These amino acids were indicated, through *in silico* analysis, as a putative endomembrane targeting signal. Only oocytes injected with the complete form of the protein exhibited a significant increase in iron content, in two iron concentrations tested, demonstrating the protein's ability to perform transmembrane iron transport.

Keywords: Iron, metal transport, NRAMP, *Oryza sativa* L., rice, *Xenopus laevis.*

Background

Metals are essential for the maintenance of living cells. They are required for many biological processes such as gene regulation and free-radical homeostasis, catalyzing electron transfer in mitochondria and chloroplast or acting as co-factors for enzymes. Since accumulation of high amounts of metals might lead to toxic effects, metal homeostasis must be tightly regulated. It has been shown that the excess of a metal such as iron, in its free form, may lead to increased generation of reactive oxygen species (ROS) through the Fenton reaction[1], and that this iron-mediated oxidative stress can cause damage to several biological macromolecules such as lipids, proteins and nucleic acids, being ultimately deleterious to cell integrity[2].

The Natural-Resistance-Associated Macrophage Protein (NRAMP) gene family was shown to play important roles in metal homeostasis. Its importance was demonstrated in several organisms, from bacteria to human[3]. Protein members of this family have a broad spectrum of specificity in metal transport including Fe, Mn, Co, Zn, Cu, Cd, Ni[4] and V[5]. Heterologous expression in yeast and molecular physiological studies in plants are some of the experiments that have characterized the family[6, 7, 8], providing information on which metals are transported and how the family is linked to metal homeostasis.

Techniques which allow high-resolution of the structures of transporters shed light on the mechanisms of ion transport, since conformational changes are part of the transport mechanism[9]. However, typical eukaryotic cells have a large number of transporters in the plasma membrane, and studying the transport mechanism of a single specific transporter is a hard task. Thus, important information about transporter proteins has come from studies of metal transporters expressed in *Xenopus laevis* oocytes[6, 10, 11, 12, 13]. One of the advantages of this method is that there is little background transport activity to interfere with the characteristics of the foreign protein and the large cell size facilitates the analysis[14].

Our group identified eight *NRAMP* gene family members in the rice genome[15]. In a recent work, we found significant negative correlation between *OsNRAMP7* (LOC_Os12g39180) expression in flag leaves during grain filling and final Fe and Zn concentrations in the grain[16]. The influence of OsNRAMP7 in metal allocation to the grain could also be supported by the co-localization of the gene with a QTL that explains around 14% of the phenotypic variation in Fe [17] and Zn concentration[18] in the grain.

Based on these previous findings, we hypothesized that the OsNRAMP7 protein is able to perform iron transport in rice plants, and in the present study we further investigated this possibility.

Results and Discussion

An *in silico* analysis of the OsNRAMP7 protein was conducted to help investigate some of its properties and its anchorage in the membrane. The topology algorithm HMMTOP (http://www.enzim.hu/hmmtop/) predicted the existence of twelve significant putative transmembrane domains (TMDs) (Fig. 1a), consistent with findings on other members of the family, which show from 10 to 12 TMDs[3]. Conserved regions, such as the signature sequence of the NRAMP family, DPGN (Asp-Pro-Gly-Asn) and the MPH motif (Met-Pro-His)[19,20] are also present in OsNRAMP7. When analyzing the protein for the presence of a transport motif to define NRAMP family membership, OsNRAMP7 shows the Consensus Transport Motif (CTM)[19,20] at the hydrophilic loop between TMDs 7 and 8 (Fig. 1). This conserved motif is a feature present in all members of the NRAMP family in rice (Supplementary Figure 1). The rice NRAMP7 protein shares important features with the isoform I of the mammalian DCT1[3], the first member of the NRAMP family to have its role in metal homeostasis described[10]: both proteins share the same number of TMDs (12) and the same number of amino acids (18) at the C-terminus domain located after TMD12 (Fig. 1a).

A three-dimensional representation of the protein was constructed (Fig. 1b-f) where all three conserved regions are in evidence (Fig. 1c-f). It is possible to observe that that the CTM is present in the protein's widest loop (Fig. 1c) and adjacent to a large cavity (Fig. 1c-d). Also, among the cavities present, that seems to be the largest. This could be evidence of a structural property of this feature. Adjacent to this structure are the DPGN and MPH motifs (Fig. 1e-f). Structures as the DPGN site are likely to be involved in divalent metal (Me^{+2}) uptake, since it bears analogy to a known functional signature for Me^{+2} transport[21]. In *E. coli* MntHAsp mutants, it was shown that the DPGN site is also implicated in Me^{+2} binding and coupling of Me^{+2} transport to proton-motive force[22]. The MPH motif participates in pH-dependent regulation by deprotonation, which could favor a conformation that enables Me^{+2} transport[22].

The localization and orientation of the DPGN and MPH motifs (seen in Fig. 1f) are shared by several cation transporter families[23]. Another important feature involving these motifs is the formation of a pair of extended peptides by their anti-parallel orientation, interrupting the TMDs. This characteristic is present in transporter structures, and the presence of discontinuous TM helices with extended peptides seems to be directly correlated with the ion transport reaction[22, 24].

OsNRAMP7 expression was observed in all rice organs evaluated. When analyzing hydroponically grown rice plants, expression in leaves was almost eight times higher of what was found in roots (Fig. 2a). In field grown plants, organs involved in grain filling (panicles and flag leaves) presented higher expression of *OsNRAMP7* then vegetative organs (stem and non-flag leaves) at the grain filling stage (Fig. 2b). Previous experiments revealed that *OsNRAMP7* expression was not altered by plant treatments leading to Fe excess or Fe, Zn or Mn deficiency, both in roots and shoots (Ricardo Stein and Marta Spohr, personal communication).

The protein sequence of OsNRAMP7 has high identity to *Arabidopsis thaliana*'s AtNRAMP3 and AtNRAMP4 (68%, Supplementary Figure 2a). All three proteins also belong to the Group II in a phylogenetic analysis (Supplementary Figure 2b). Such similarity is relevant since these proteins were demonstrated to be involved in Fe transport by complementing a mutant strain of yeast defective in Fe uptake[7] and, also, by mediating iron transport in Arabidopsis, being involved in iron efflux from vacuoles during germination[8]. AtNRAMP3's subcellular localization on the vacuolar membrane[25] suggests a function in intracellular metal homeostasis. Other more distantly related NRAMP proteins have been shown to transport Al[26]. Although recent publications presented phylogenetic trees of the rice NRAMPs[27, 28], none were complete, not including all members of the family.

To test the ability of the OsNRAMP7 protein to mediate transmembrane iron transport, we expressed the protein in *Xenopus* oocytes. Before starting the experiments, we investigated the OsNRAMP7 protein sequence for the presence of a possible targeting signal peptide cleavage site using the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/). The presence of a targeting sequence could impair targeting of the protein to the plasma membrane in heterologous expression systems like *Xenopus* oocytes. SignalP described a possible targeting signal peptide, with a maximum cleavage site probability between amino acids 16 and 17. Targeting sequences were removed in previous reports, as for expression of chloroplastic membrane proteins (e.g. Mariscal et al.[29]; Maughan et al.[30]). For this reason, not only the full length cDNA of *OsNRAMP7* was used for oocyte expression, but also the N-terminal truncated form, missing the first 15 amino acids of the protein.

A barley aquaporin mRNA was used as a control in order to test for the quality of the injections and translation of the injected RNA into protein[31]. A simple swelling assay can be used in these positive control experiments. A second control group, of oocytes injected with

water, was also used. Therefore, five groups of oocytes were analyzed: uninjected oocytes, oocytes injected with water, aquaporin, *OsNRAMP7* and the truncated form of *OsNRAMP7*. The optimal timing for protein expression was established by exposing aquaporin mRNA injected oocytes to swelling assays (where they were exposed to 50% MBS) 3, 4 or 5 days after injections. The functional aquaporin protein resulted in the oocytes swelling more rapidly (Fig. 3a). These experiments established that a four day incubation period was optimal for expression of the aquaporin. Hence, four days after mRNA injections oocytes from all treatments were exposed to MBS medium containing 50 or 100 μM $FeCl_3$, where they were kept for 24 hours. These iron concentrations are used in hydroponic solutions for rice growth experiments[26].

Single cells were examined for the absorption of iron by quantification of total iron content. A significant increase of iron content in oocytes injected with the full-length form of *OsNRAMP7* was observed (Fig. 3b), while cells that were not injected or injected with water, aquaporin or the truncated form of *OsNRAMP7* did not show a significant increase of iron content (Fig 3b). The presence of the functional form of the protein OsNRAMP7 resulted in increased iron uptake.

These results suggest that the cleaved fraction of the OsNRAMP protein may be essential for function and not necessarily an endomembrane targeting signal. The lack of function of the truncated form of OsNRAMP7, when compared to the complete form, may be explained by a possible change in conformational structure of the protein due to the alteration of the N-terminal portion[32, 33], which could impair its activity. Previous studies showed that, in certain proteins, alteration by deletion of a fraction of the N-terminal portion might lead to decreased activity or even, as seen here, to protein loss of function[33, 34].

Conclusions

The increased iron content in oocytes upon expression of OsNRAMP7 indicates that this protein is able to mediate iron transport. Together with its high similarity to other NRAMP proteins shown to be iron transporters and its high expression in reproductive organs, the presumed function of *OsNRAMP7* in iron homeostasis during grain filling is confirmed. The protein could have a role in the internalization of metals in vacuoles, resulting in lower metal availability to be transported to panicles. Therefore, it is possible to confirm an active role of OsNRAMP7 in iron homeostasis. Further studies, such as sub-cellular localization through Green Fluorescent Protein (GFP) fusion and complementation of yeast strains defective in metal transport, are being conducted in order to better understand the role of this transporter in metal homeostasis in rice plants.

Materials and Methods

For the analysis of panicles, stem, flag leaf and other leaves, rice plants (*Oryza sativa* L. cv. Nipponbare) were field-grown as described[16] up to the reproductive stage 5 (R5) according to the classification established by Counce et al[35]. For analysis of OsNRAMP7 expression in roots and leaves, seeds were germinated for 4 days in an incubator (28° C, first 2 days in the dark and last 2 days in the light) on paper soaked with distilled water. After germination and growth in vermiculite and nutrient solution (0.1 mM KCl, 0.1 mM KH_2PO_4, 0.7 mM K_2SO_4, 2 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.5 mM $MgSO_4 \cdot 7\ H_2O$, 0.5 µM $MnSO_4 \cdot 4\ H_2O$, 0.01 µM $(NH_4)_6\ Mo_7\ O_{24} \cdot 4\ H_2O$, 10 µM H_3BO_3, 0.5 µM $ZnSO_4 \cdot 7\ H_2O$, 0.2 µM $CuSO_4 \cdot 5\ H_2O$, 100 µM $FeSO_4 \cdot 7\ H_2O$, 100 µM EDTA) for 14 days (28° C, with 16 h of light), plants were transferred to pots containing 2.5 L of nutrient solution. All solutions were replaced every 3 days. Plants were cultivated in a growth room at 26 ± 1° C under white light with a photoperiod of 16/8 h light/dark cycle (irradiance of approximately 100 µmol $m^{-2}s^{-1}$ at the

plant tops) for another period of 14 days, when analyses were conducted. RNA extraction was performed with pooled material from three plants in each replicate.

Total RNA was extracted using Concert® (Invitrogen Life Technologies) reagent. First-strand cDNA synthesis was performed after DNAse treatment with reverse transcriptase (M-MLV®, Invitrogen Life Technologies) using 1 μg of RNA, quantified using Qubit® (Invitrogen Life Technologies). An Applied Biosystem 7500 real-time cycler was used to carry out the qRT-PCR analysis. A 240 bp region was amplified using gene-specific primers (5'-GCTGCCAAATCAGATCATCA-3' e 5'-GCTTCAGGACGACACAGTCA-3'). Reaction settings were performed according to Sperotto et al[36]. Gene expression was evaluated by the $2^{-\Delta CT}$ method[37, 38]. Each data point corresponds to three biological replicates, which were analyzed in four technical replications. A ΔC_T value was obtained by subtracting the Ubiquitin C_T value from the C_T obtained for the gene of interest. When appropriate, data were subjected to analyses of variance (ANOVA) and means were compared by the Duncan test ($P \leq 0.05$) using the SPSS Base 17.0 for Windows (SPSS Inc., USA).

Three-dimensional structure of OsNRAMP7 was built using homology modeling, since its crystal structure is not available. The protein sequence (Accession No. NP_001067135, length: 541 amino acid) was retrieved from NCBI GenBank. A 3D model based on multiple-threading alignments by LOMETS and iterative TASSER assembly simulations was built at the I-TASSER webserver[39]. The comparison between template and target sequence resulted in C-score equal to -3.74, TM-score of 0.39±0.13 and RMSD of 14.5±3.7Å. The pre-refined model was examined under the Ramachandran plot and the residue properties plot was evaluated using the PROCHECK server[40]. Final adjustments and molecular visualization were performed using PyMOL (http://www.pymol.org).

Two forms of *OsNRAMP7* cDNA were produced by PCR, one corresponding to the full coding region of *OsNRAMP7* cDNA and one where a putative signal peptide present on

the N-Terminal portion of the protein was removed. The full coding region was amplified using two specific primers (Forward 1, 5'- AATAGATCTACCCCCATCTCCAAATC-3'; Reverse, 5'- AATGGTACCTGAAGCGTATAGTTGTG-3'). The amplification product of the truncated version of *OsNRAMP7* was obtained using a different forward primer (Forward 2, 5'- AGATCTATGGCCACCCCCGCC-3'), while maintaining the same Reverse primer. A BglII restriction site was added at the 5' end of both forward primers. The resulting PCR product was cloned into the *Xenopus laevis* oocyte expression vector pT7TS' BglII and EcoRV sites. Plasmid DNA was digested with EcoRI, and mRNA was produced with the Ambion *In Vitro* Transcription kit (mMessage mMachine Ultra®) following the manufacturer's instructions. Oocytes were prepared as previously described[41]. A 50 ng aliquot of mRNA in 50 nl of water was injected into stage V and VI oocytes obtained from *X. laevis*. The oocytes were incubated in Modified Barth's Saline (MBS) (8.8 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, $CaCl{\cdot}2H_2O$) at 20° C for 4 days. Swelling tests were carried out by transferring to 0.82 mM $MgSO_4{\cdot}7H_2O$, 0.33 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.41 mM $CaCl{\cdot}2H_2O$ at 20°C for 4 days.

Swelling tests were carried out by transferring oocytes to 50% MBS (MBS 1:1 water), and time lapse images were taken every five minutes with a digital camera attached to a dissection microscope[31]. The swelling rate was established by comparing the mean diameter measure of six independent oocytes, for four times each, during a twenty minute interval. Iron uptake assays were performed on oocytes four days after the mRNA injections, when they were incubated in MBS solution containing 50 or 100 µM $FeCl_3$, added of Ascorbic Acid in an equimolar concentration to maintain the Fe in an available form[5] or maintained in the MBS solution without the addition of iron. Oocytes were kept in these solutions for 24 hours before uptake analysis. After the incubation period the oocytes were washed in DCB solution (0.03 M $Na_3C_6H_5O_7{\cdot}2H_2O$, 0.125 M $NaHCO_3$ and 0.06 M $Na_2S_2O_4$[42]), prepared in MBS for 10

minutes under slight agitation to remove any iron that might have been deposited on the plasma membrane. The colorimetric ferrozine-based assay for Fe^{2+} quantification was performed as described[43]. Measurements were conducted in 10 single oocytes for each treatment. Data was subjected to statistical analysis using Student's *t* test ($P = 0.05$) on the SPSS Base 12.0 for Windows (SPSS Inc., USA).

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Authors' contributions

JPF conceived the work. LSS and JPF performed initial studies. LSS performed sequence analyses, RT-qPCR assays, DNA constructions and 3D modeling. LSS and AJM performed the experimental procedures with *Xenopus* oocytes and analyzed the data. LSS drafted the original manuscript. JPF was responsible for the final editing. All authors read and approved the final manuscript.

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Legends to figures

Figure 1: (a) Schematic representation of OsNRAMP7 protein constructed with the software TMRPres2D[44]. The predicted 12 TMDs topology is based on HMMTOP algorithm. The conserved motifs DPGN and MPH and the putative transit peptide are indicated. (b) Schematic representation of transport in the three-dimensional model of OsNRAMP7 depicted by the secondary structure. (c) and (d) Transversal view of OsNRAMP7. In (d) the asterisk indicates a large cavity present in the protein. (e) Parallel view to the membrane with highlighted structures. (f) Detail of conserved structures. (c – f) Extended peptides MPH and DPGN are colored yellow and magenta, respectively. CTM is depicted in red.

Figure 2. (a) Relative expression levels (RT-qPCR, relative to Ubiquitin expression) of *OsNRAMP7* in hydroponically grown 28-day old rice plants. (b) Relative expression levels of *OsNRAMP7* in distinct rice plant organs harvested at the R5 (grain filling) stage from field-grown plants. Values are averages of three replicates ± SE. Means indicated by different letters in (a) are different by *t*-test ($P \leq 0.05$), while in (b) they differ by the Duncan test ($P \leq 0.05$).

Figure 3. Plant mRNA expression assays in *Xenopus laevis* oocytes. (a) Swelling assay of aquaporin injected oocytes. Six independent oocytes injected with aquaporin mRNA were analyzed on three consecutive days starting at the third day after the injections, when they were subjected to 50% MBS and had the diameter size determined five times during twenty minutes to establish an increase in volume. (b) Relative variation of iron content in single oocytes injected or not with mRNA (aquaporin, OsNRAMP7 and truncated OsNRAMP7) when exposed to two different concentrations of iron in MBS solution. Values are the average

of ten single oocytes and data was subjected to statistical analysis by Student's *t* test (P = 0.05).

Supplementary data

Supplementary Figure 1: Alignment of members of the NRAMP family of rice made with ClustalW (http://www.clustal.org/). Conserved amino acids are shaded and the Consensus Transporter Motif (CTM) is highlighted in the box. Residues marked in darker shades of grey belong to a conserved group of amino acids defined as strong by ClustalW.

Supplementary figure 2: (a) Alignment of AtNRAMP3, AtNRAMP4 and OsNRAMP7 made with ClustalW (http://www.clustal.org/). Conserved amino acids are shaded. 68% of the amino acids are identical in the three proteins. (b) Phylogenetic tree of NRAMP members in *Arabidopsis* and rice. Sequences we aligned and the tree constructed using the PhyML's maximum likelihood method[45] computed by Seaview[46].

Figures

Figure 1

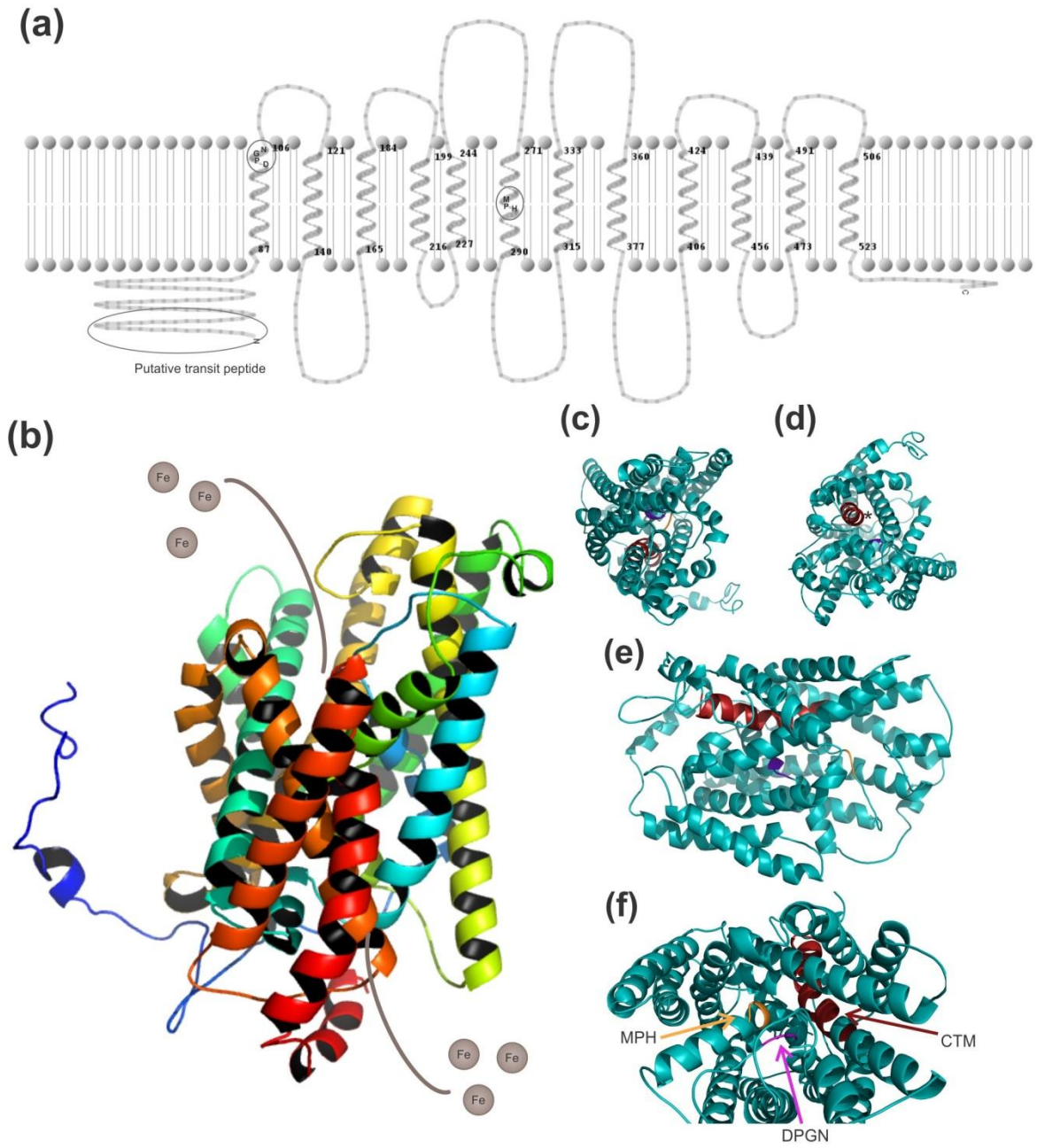
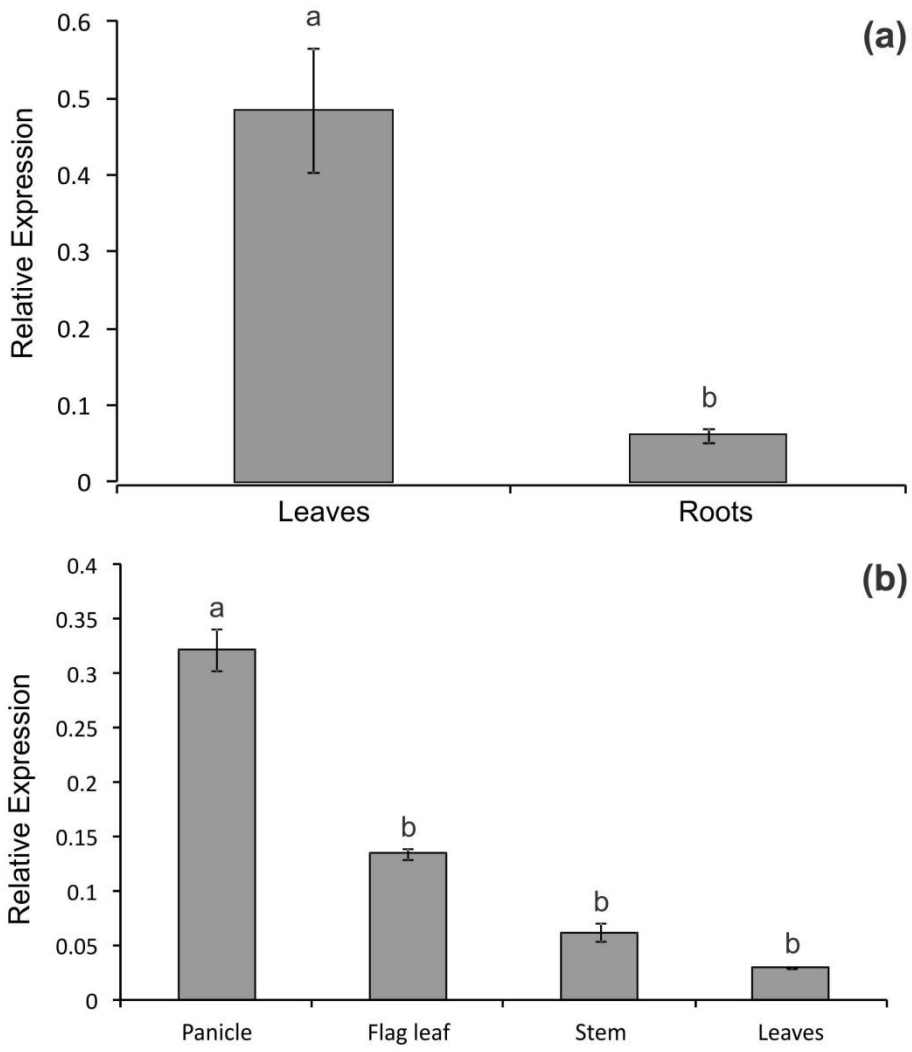


Figure 2


(a)
Relative Expression
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
Leaves
Roots
(b)
Relative Expression
0.4
0.35
0.3
0.25
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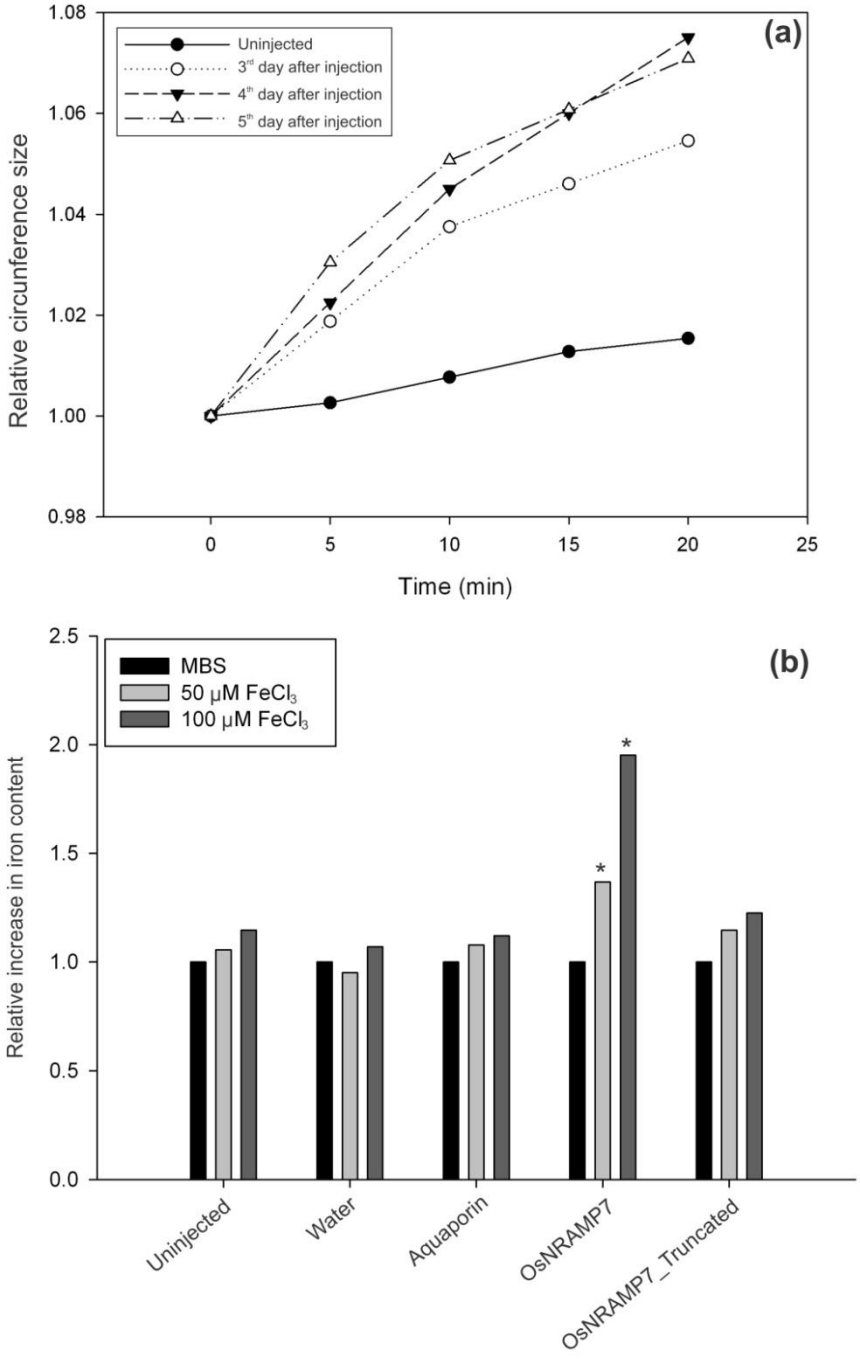
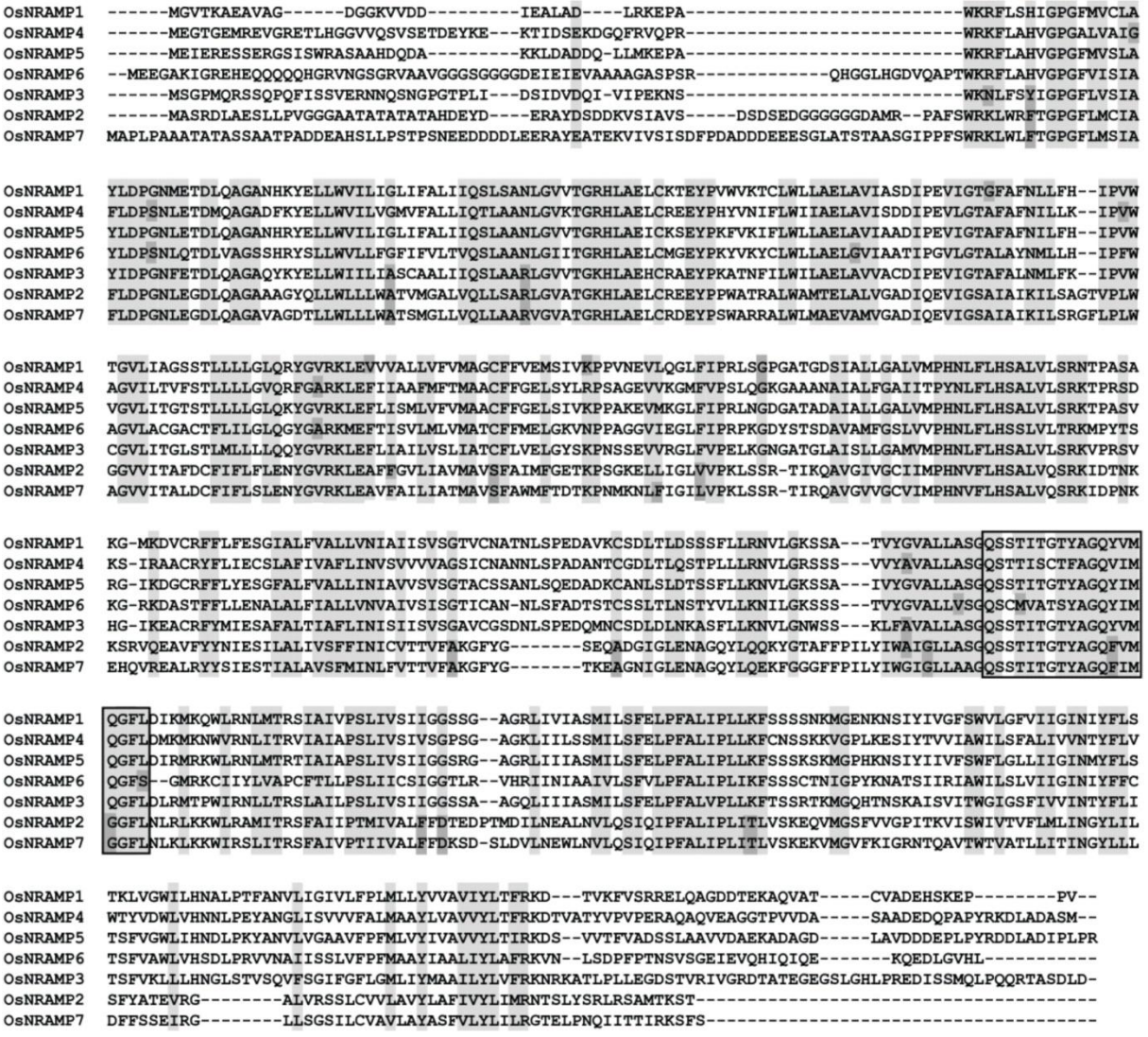
b
b
Panicle
Flag leaf
Stem
Leaves

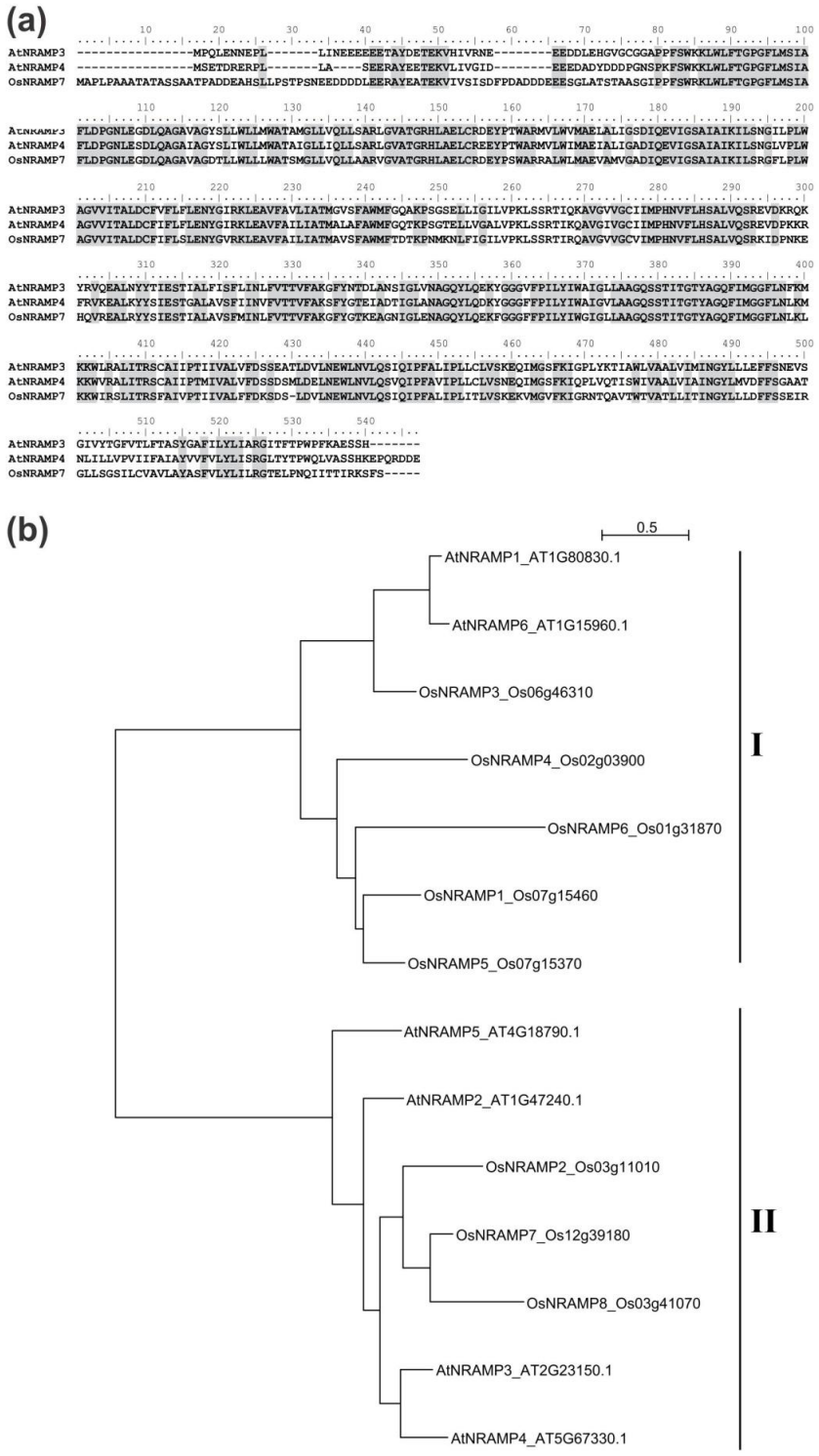
Figure 3


(a)
Uninjected
3rd day after injection
4th day after injection
5th day after injection
Relative circunference size
1.08
1.06
1.04
1.02
1.00
0.98
0
5
10
15
20
25
Time (min)
(b)
MBS
50 µM FeCl3
100 µM FeCl3
Relative increase in iron content
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Uninjected
Water
Aquaporin
OsNRAMP7
OsNRAMP7_Truncated

Supplementary figure 1



Supplementary figure 2



Capítulo 3

Dual impact on rice plants bearing *OsFer2* mutation

Dual impact on rice plants bearing *OsFer2* mutation

Lívia Scheunemann dos Santos, Luiza Monteavaro Mariath, Diogo Ribeiro Demartini, Ricardo José Stein, Janette Palma Fett.

Abstract

Iron is an important micronutrient to plants, involved in several biological processes. However, it is important that plants maintain control of iron homeostasis, since excess of iron in the free form may catalyze the formation of oxygen radicals. One of the strategies to avoid leaving iron available for such reactions is to store Fe inside ferritins. Ferritin is a spherical protein capable of storing iron in its core, also acting as an iron buffer in cells. In this study, a mutant line with impaired expression of *OsFer2* was used to investigate a possible role of ferritins in iron overload responses. Although rice has two genes encoding ferritin, the *OsFer2* mutation resulted in decreased ferritin transcript levels of both genes and less protein. As mutant plants accumulated less biomass than WT also under the control treatment, rice ferritin is apparently important for iron homeostasis and plant development even when iron availability is considered normal. No photo-oxidative damage could be observed through maximal efficiency of PSII photochemistry in mutant plants under iron excess conditions. However, shoots of mutant plants had higher content of malondialdehyde (MAD), an indicative of lipid peroxidation, and roots of mutant plants had increased APX activity, both in the iron excess treatment, indicating a possible enhancement of oxidative stress in the mutant under this condition. Further studies are required to establish if increasing iron stress would further alter the oxidative stress response or if a compensating mechanism, such as influx to vacuole and production of frataxin, are involved in oxidative stress responses in *OsFer2* rice mutants.

Keywords: ferritin, *Oryza sativa* L., oxidative stress.

Introduction

Iron is an essential nutrient for plant growth and development. Both physical and chemical properties of iron enable this micronutrient to participate in most basic reduction reactions in an organism. Iron is essential for a variety of processes such as photosynthesis and DNA synthesis (Briat & Lobréaux, 1997). Iron is directly involved in the electron transfer chain of respiration and acts a co-factor of enzymes involved in the synthesis of plant hormones (Guerinot, 2011). Since iron homeostasis must be tightly regulated, plants have different strategies to maintain cellular homeostasis. Among these strategies are allocation of the metal to the vacuole, translocation to plastids and storage in ferritins.

Different functions have been described to ferritins depending on the species analyzed. In peas it seems to have an important role in iron storage in seeds, releasing the metal when necessary and enabling germination (Becker et al., 1998). A distinctive role has been proposed to *Arabidopsis* seeds, since it is estimated that no more than 5% of the seed iron content would be stored in ferritins (Ravet et al., 2009).

Ferritins have been found in mitochondria, chloroplasts and cell walls (Becker et al., 1998). The functional ferritin protein is formed by 24 subunits, which can store up to 4000 ferric iron atoms each. Before being stored inside the mineral core of the protein as Fe^{3+}, iron is oxidized by the protein ferroxidase center (Arosio et al., 2008). Ferritins provide an intracellular easy-access form of iron and might have a putative detoxification property. This could be due to the fact that the oxidation reaction necessary for iron storage actually consumes oxygen and hydrogen peroxide (Arosio et al., 2008).

Two ferritin genes have been identified in rice, *OsFER1* and *OsFER2* (Gross et al., 2003). Very little sets these two genes apart. *OsFER1* possesses three deletions and 15 single nucleotide changes within the coding region (Stein et al., 2009). Using specially designed primers, Stein et al. (2009) was able to separate by electrophoresis the transcripts amplified from each gene.

The present work uses a mutant *OsFER2* line to study ferritin influence in protection against oxidative stress in rice. This mutant is a result of the insertion of the *Tos17* transposon, one of the most active retroelements in rice plants.

Results

A rice line containing a *Tos17* insertion in the *OsFER2* gene was identified in the Rice Genome Resource Center (RGRC) bank. Seeds were obtained, plants segregating for the mutation were screened by PCR analysis using specific primers and an homozygous line was identified. Aligning the genomic sequence of *OsFER2* with the respective Flanquing Sequence Tags (FSTs) of the mutant line NG0250 allowed to determine that the insertion of the retroelement occurred in the 3'UTR (untranslated region) of the gene (Figure 1).

There was the necessity to determine if the gene was being transcribed and the protein produced even in the presence of the insertion. For that, the amount of transcripts of both ferritin genes was analyzed. The expression pattern of the ferritin genes in both wild type (WT) and mutant lineage were evaluated by semi-quantitative RT-PCR, using an ubiquitin gene as control for loading and optical density measurements. It was possible to observe that the level of ferritin transcripts (*OsFER1* and *OsFER2*) was higher on wild type plants under iron excess, while in mutants a low amount of transcripts was detected in either treatment (Figure 2a).

Using an optical density analysis it was possible to observe an increase by two fold in the amount of *OsFER1* transcripts from the control to iron excess treatment in WT plants (Figure 2a). An increase of 20% in the amount of *OsFER2* transcripts was also observed in WT plants. Mutant plants had fewer transcripts in both treatments. Under control conditions, mutant plants had 25% less *OsFER1* transcripts than WT. When subjected to iron excess, mutant plants increased the level of *OsFER1* transcripts by 30%, while WT plants more than doubled the *OsFER1* transcript abundance. The level of *OsFER2* transcripts in mutant plants was not sufficient to allow optical density analysis (Figure 2a).

To confirm that mutants were impaired in ferritin protein production, a Western Blot analysis was performed. Only shoots of plants subjected to iron excess treatments were used for this analysis (when more protein would be produced and, therefore, more easily detected). Virtually no protein was detected in mutant plants (Figure 2b).

Once established that the mutant plants were in fact defective in the production of ferritin, stress-related analysis were performed in order to assess its role in protection against oxidative damage. Chlorophyll fluorescence was evaluated to establish if the iron excess treatment had affected the photosynthetic apparatus. Plants with low content of ferritin did not have a significant difference of the maximal efficiency of PSII photochemistry (Fv/Fm)

values when compared to WT plants under the same conditions (Figure 3). No significant difference between treatments was observed in WT plants (Figure 3). Other measurements related to photosynthetic efficiency, such as the quantum yield of electron transport (ET0/ABS) and the efficiency with which an electron can move to the PSI electron acceptor (ET0/TR0) also didn't result in significant differences between genotypes or treatments (data not shown).

Roots of mutant plants showed a significant decrease in length when subjected to iron stress treatment (Figure 4). The same was not observed in WT plants. Although there was no difference in shoot length, either between genotypes or treatments (Figure 4), there was a significant difference in shoot fresh weight. While WT plants showed a significant decrease in fresh weight under iron excess, the same could not be observed in mutant plants (Figure 5a). Fresh weight of mutant plants was significantly smaller than in WT in the control condition, however was not altered by the iron excess treatment. The same pattern was observed for the fresh weight of roots (Figure 5b).

There was no difference in shoot dry weight either among genotypes or treatments (Figure 5c). On the other hand, there was a significant decrease in root dry weight in WT plants under iron excess. Despite not showing a significant difference between treatments, root dry weight of mutant plants was much lower in control condition than in WT plants (Figure 5d).

Both shoots and roots were used for oxidative damage analyses. Oxidative damage to lipids was observed through malondialdehyde (MDA) quantification. MDA is produced when polyunsaturated lipids are degraded by reactive oxygen species (ROS) and can be quantified by Thiobarbituric Acid Reactive Substances (TBARS) analysis. Both genotypes increased MDA content when subjected to iron excess (Figure 6a). However, shoots of mutant plants seemed to accumulate more MDA than WT plants under excess iron (Figure 6a). A tendency of increase in MDA concentration was also observed in roots of mutant plants (Figure 6b). Despite a tendency of raise in H_2O_2 levels in roots of mutant plants under iron excess treatment, no significant difference was observed in H_2O_2 assays (Figure 6c, d).

There was no significant difference in shoot ascorbate peroxidase (APX) activity (Figure 7a), while roots of both genotypes had increased APX activity under iron excess (Figure 7b). Of these, mutant plants had higher increase in APX activity than WT plants. There was no difference in catalase (CAT) (Figure 7c, d) or superoxide dismutase (SOD)

(Figure 7e, f) activity in roots and shoots of both genotypes.

Discussion

Rice mutants with lower content of the ferritin protein accumulated less biomass than WT plants. This can be stated by the fact that, despite having similar shoot lengths, shoot weight of mutant plants were significantly lower than those observed in WT under control conditions. This difference is even stronger when analyzing their roots. Although WT and mutant plants had no significant difference in length, WT roots were much more abundant, resulting in higher fresh weight.

Mutant plants accumulated less biomass than WT plants in the control treatment: lower fresh weight in roots and shoots, lower dry weight in roots. These results indicate that, besides a possible function in prevention of oxidative stress when plants are exposed to iron excess, ferritins may also have an important role in iron homeostasis under normal iron availability.

Both genotypes presented similar levels of peroxide production and antioxidant enzyme activities when subjected to iron excess treatment. However, in the mutants, APX activity in roots under iron excess was more evident. The increased MDA concentration in shoots of mutant plants submitted to iron excess could be a sign of oxidative stress. The combined results observed in *OsFER2* mutant plants indicated that ferritin might have a protective role related to iron stress in rice plants.

It has been described that different organs might have distinct physiological strategies to respond to oxidative stress. In a work conducted by Fang et al. (2001) iron stress resulted in decreased SOD activities, but increased APX activity in leaves. On a different note, Bode et al. (1995) reported that APX activities under iron stress were unchanged. In a different experiment, Majerus et al. (2007) described that sheaths and laminae respond differently to iron stress. On their experiment, SOD activity was stimulated in the first and APX in the latter. Variations in ferritin concentration among different plant organs could have an indirect impact on the activities of these enzymes, since levels of free iron would be altered according to ferritin availability.

Plants can resort to several detoxification strategies. The vacuole has an important role when considering intracellular iron homeostasis. In *Arabidopsis*, AtFPN2 (Morrisey et al.,

2009) and AtVIT1 (Kim et al., 2006) were considered as iron influx proteins to the vacuole. Since a vacuolar influx transporter of iron in rice is not known, it is virtually impossible to determine if a particular protein with this function is up-regulated in this situation to overcome the stress generated by iron overload.

Also involved in subcellular iron homeostasis is the mitochondrial protein frataxin. Putative functions of this protein include assisting in iron–sulfur cluster assembly (Chen et al., 2002) or involvement in energy conversion and oxidative phosphorylation (Ristow et al., 2000). This protein is believed to act in conjunction with ferritins and the vacuole to prevent oxidative stress. Frataxin mutants showed increase in the content of reactive oxygen species and high levels of transcripts that encode proteins involved in oxidative stress response (Busi et al., 2006). This supports the proposed role of the protein on prevention of oxidative damage.

There might be an increase in the production of proteins such as frataxin or a vacuole iron influx transporter in rice plants bearing *OsFER2* mutation. This compensating mechanism could be set into action in the presence of low amounts of ferritin, protecting the plant from oxidative damage. Further studies in order to better understand iron homeostasis and transport to the vacuole and in organelles such as chloroplasts and mitochondria will lead to a better understanding of the mechanisms plants may resort to when under abiotic stress. While knowledge in this area is still at surface, research in oxidative stress responses may lead to a better understanding of possible compensating mechanisms.

It appears that ferritins act in iron homeostasis in rice plants in two ways: one independent from the iron status inside the cell and a second one in response to iron excess. Plants with impaired ferritin production accumulated less biomass than WT in the control treatment and had increased stress responses (such as MDA concentration in shoots and APX activity in roots) under iron stress. Exposing plants to an even higher concentration of iron may help to further characterize ferritin involvement in oxidative stress responses in rice.

Material and Methods

Plant growth

Seeds of the rice (*Oryza sativa* L. cv. Hwayoung) mutant line NG0250, with a *Tos17* insertion in the *OsFER2* gene, were requested from the Rice Genome Resource Center – RGRC (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/index.html.em). Homozygous lines were identified by PCR using specific primers (F: 5' ACTTGCCAGGCTTCGAGTTA 3', R: 5' CGCAGTAGCAATGGAGTGAA 3', *Tos17* 3' tail: 5' AGGTTGCAAGTTAGTTAAGA 3'). Experiments were conducted with homozygous plants of this line and the WT background line.

Seeds were germinated for 4 days in an incubator (28° C, first 2 days in the dark and last 2 days in the light) on paper soaked with distilled water. After germination and growth in vermiculite and nutrient solution (0.1 mM KCl, 0.1 mM KH_2PO_4, 0.7 mM K_2SO_4, 2 mM $Ca(NO_3)_2 \cdot 4\ H_2O$, 0.5 mM $MgSO_4 \cdot 7\ H_2O$, 0.5 µM $MnSO_4 \cdot 4\ H_2O$, 0.01 µM $(NH_4)_6\ Mo_7\ O_{24} \cdot 4\ H_2O$, 10 µM H_3BO_3, 0.5 µM $ZnSO_4 \cdot 7\ H_2O$, 0.2 µM $CuSO_4 \cdot 5\ H_2O$, 100 µM $FeSO_4 \cdot 7\ H_2O$, 100 µM EDTA) for 14 days (28° C, with 16 h of light), plants were transferred to pots containing 2.5 L of nutrient solution. All solutions were replaced every 3 days. Plants were cultivated in a growth room at 26 ± 1° C under white light with a photoperiod of 16/8 h light/dark cycle (irradiance of approximately 100 µmol $m^{-2}s^{-1}$ at the plant tops) for another period of 14 days. After this period they were subjected to iron excess treatment, were 1.25 mM of $FeSO_4$ was the final concentration of iron in solution. To maintain iron solubility an equimolar concentration of EDTA was added to the solution (Sillanpää & Oikari, 1996). Solutions were changed every three days and plants were under this condition for 9 days, when analyses were conducted.

RNA extraction and cDNA synthesis

Rice leaves were harvested from plants grown under iron excess or control conditions for 14 days (n = 4 per each group). RNA extractions and cDNA synthesis were performed as described by Sperotto et al (2010).

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR was conducted as described by Stein et al (2009) with the following modification: amplification of the ubiquitin cDNA was used to normalize the data. Analysis was performed using three biological replicates, each consisting of three plants. Ubiquitin optical density was designated 1.00 and the relative normalized optical density for the remaining sample was expressed as percentage. The signal intensity of the stained bands was photographed using a digital imager (Kodak DC120 Zoom Digital Camera) and analyzed using the ImageJ 1.45 program (http://rsbweb.nih.gov/ij/index.html). Although only one representative gel was shown, the quantified data represents the averages of three independent replicates.

Protein extraction and Western Blot

Protein extraction was performed as described by Motta et al. (2001) with modifications. 1 ml of extraction buffer (50 mM Tris pH 8, 10% sucrose, 1 mM EDTA, 1 mM PMSF, 1 mM θ-phenanthroline, 0,1% SDS) was added to 100 mg of leaf powder homogenized with liquid nitrogen. The mixture was pelleted by centrifugation at 10000 rpm for 10 minutes at 4° C. The supernatant was transferred to 1.5 ml eppendorf tubes and protein concentration was determined using the Quant-iT Protein Assay Kit® and the Qubit® Fluorometer (Invitrogen). Samples (20 µg) were analyzed on 12.5% SDS-PAGE, according to Laemmli (1970). Western blot analysis was performed according to Silveira et al. (2009) with the following modifications: electrotransfer was used to relocate proteins to PVDF membrane (140 V, 350 mA, 1 hour) and the membrane was incubated with serum diluted in Blotto (1:200) overnight. Representative blots are shown.

Chlorophyll *a* fluorescence

Chlorophyll fluorescence was quantified using a portable OS-30p Chlorophyll Fluorometer (Opti-Sciences Inc., Hudson, NH, USA) in a room at 28 ± 1°C under white light with a photoperiod of 16/8 h light/dark cycle (irradiance of approximately 100 µmol $m^{-2}s^{-1}$ at plant tops). The OJIP rising transient measurements were made on attached first fully expanded leaves which were dark-adapted for 12 hours. Eight plants from each genotype and treatment were used. According to the model of energy fluxes in this test, the photon flux

absorbed by the antennae pigments is indicated as absorption flux (ABS).

Part of this excitation energy is dissipated, mainly as heat and less as fluorescence emission, the excitation energy flux which reaches the reaction center (RC) and gets trapped there (in the sense of leading to QA reduction) is indicated as trapping flux (TR). In the RCs, the excitation energy is converted to redox energy by reducing primary quinine electron acceptor of PSII (QA) to QA−, which is then reoxidized to QA, leading to an electron transport flux (ET), which maintains the metabolic reactions of photosynthetic apparatus. The derivation of the formulae for the various energy fluxes and for the flux ratios in the JIP test is resultant from Strasser & Strasser (1995) and Krüger et al. (1997) (for a review, see Strasser et al., 2000).

The trapping probability (TR0/ABS, the Fv/Fm ratio) was estimated, which is the probability that an absorbed photon will be trapped by the Photosystem II reaction center with the resultant reduction of QA (primary electron acceptor of Photosystem II). Means were compared by Student's t test ($p \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., Chicago, IL, USA).

Oxidative Damage to Lipids 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 Du & Bramlage (1992). 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. Analysis was performed using three biological replicates, each consisting of three plants. Means were compared by Analysis of Variance ($p \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., Chicago, IL, USA), and no significant differences were detected.

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 & 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 concentration was determined using the Quant-iT Protein Assay Kit® and the Qubit® Fluorometer (Invitrogen). Analysis was performed using three biological replicates, each consisting of three plants. Means were compared by Tukey test ($p \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., Chicago, IL, USA) where appropriate.

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Figures

Figure 1

Figure 1: Structure of the *OsFER2* gene. Introns are indicated by black lines and exons by gray boxes. The position where the retroelement *Tos17* was inserted in the mutant line NG0250 is also indicated.

Figure 2

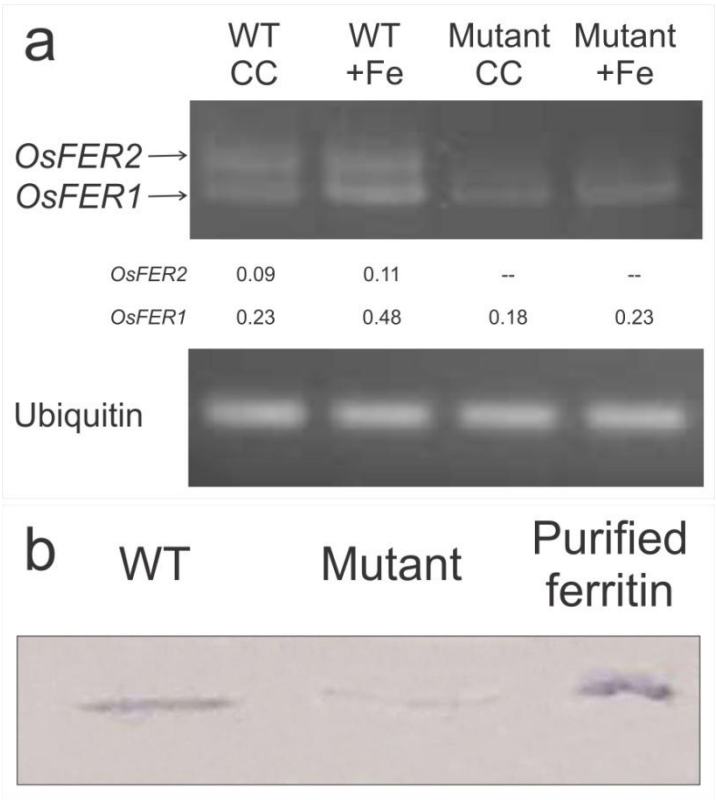

Figure 2: Analysis of ferritin protein and RNA expression in rice leaves. **a**. Semi-quantitative RT-PCR of WT and mutant plants under control (CC) or iron excess treatment (+Fe). Semi-quantitative RT-PCR was performed using standard conditions and the expression of ubiquitin (*OsUbq*) was used as control for equal loading. Numbers below each lane represent the percentage in relation to ubiquitin optical density (means of three independent replicates). **b**. Western Blot of protein extracted from WT and mutant plants submitted to iron excess treatment (1.25 mM) for nine days. Purified pea ferritin (3 µg) was used as positive control.

Figure 3

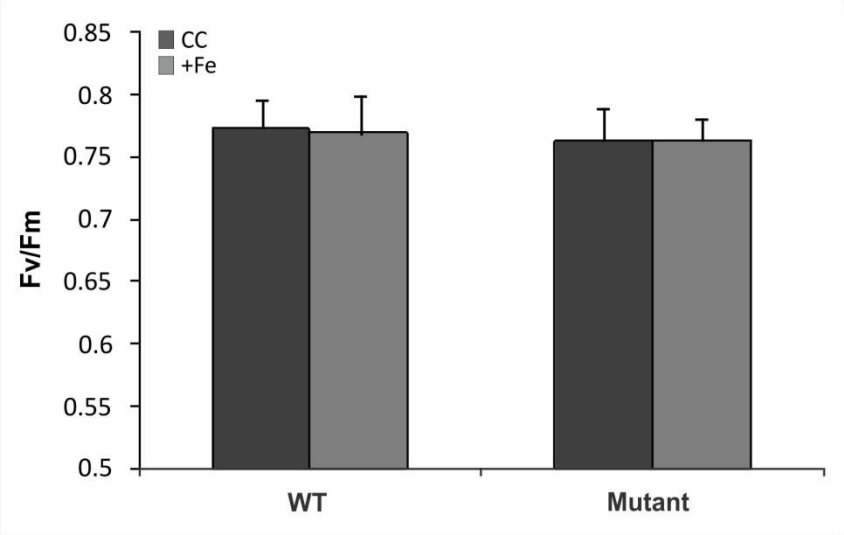

Figure 3: Analysis of maximum photochemical efficiency (Fv/Fm) on leaves of rice plants exposed for 9 days to control (CC) or iron excess treatment (+Fe). Values are the averages of five samples ± SE.

Figure 4

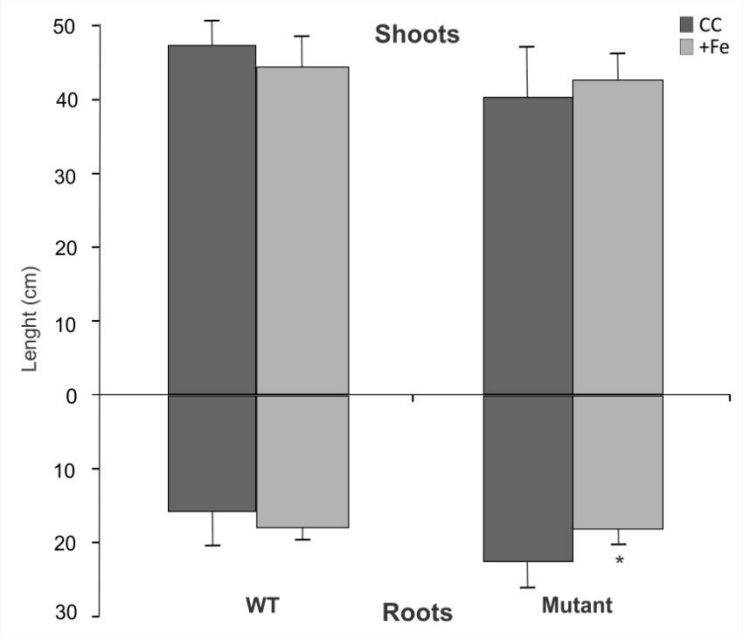

Figure 4: Shoot and root length of WT and mutant plants under control (CC) or iron excess treatment (+Fe). Values are the averages of ten samples ± SE. Statistical differences by the Student's t-test in comparison to control are shown by one asterisk ($p \leq 0.05$).

Figure 5

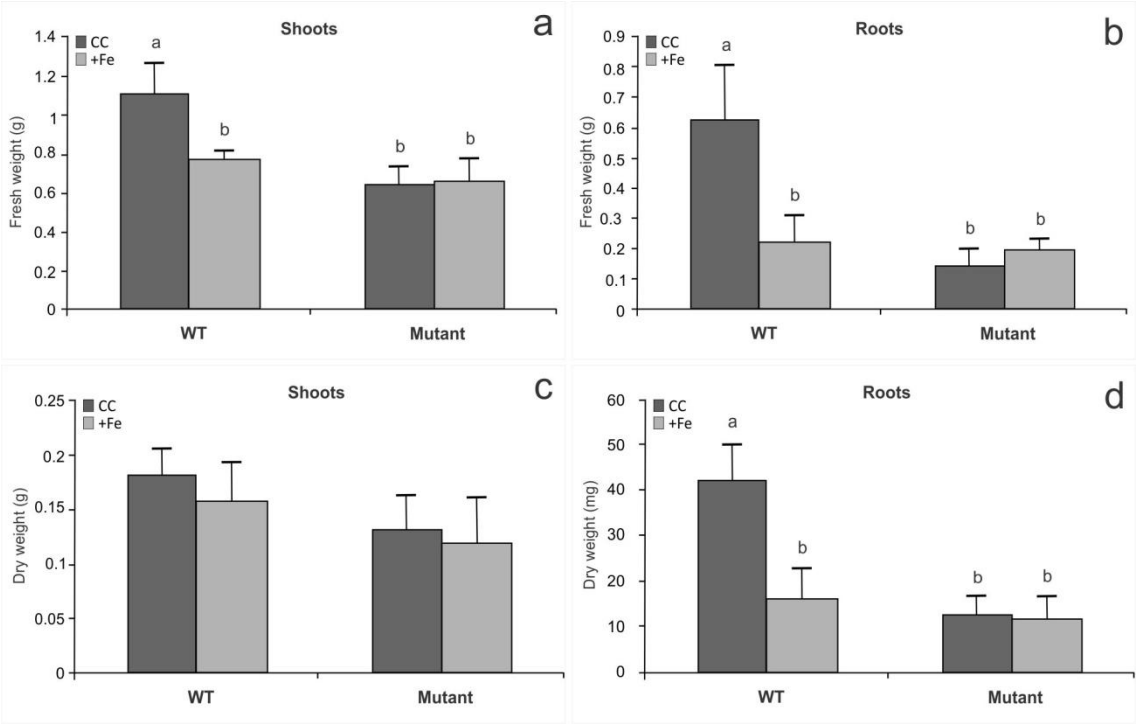

Figure 5: Fresh and dry weight of WT and mutant plants under control (CC) or iron excess treatment (+Fe). Values are the averages of ten samples ± SE. Means indicated by different letters are different by the Tukey test ($p \leq 0.05$).

Figure 6

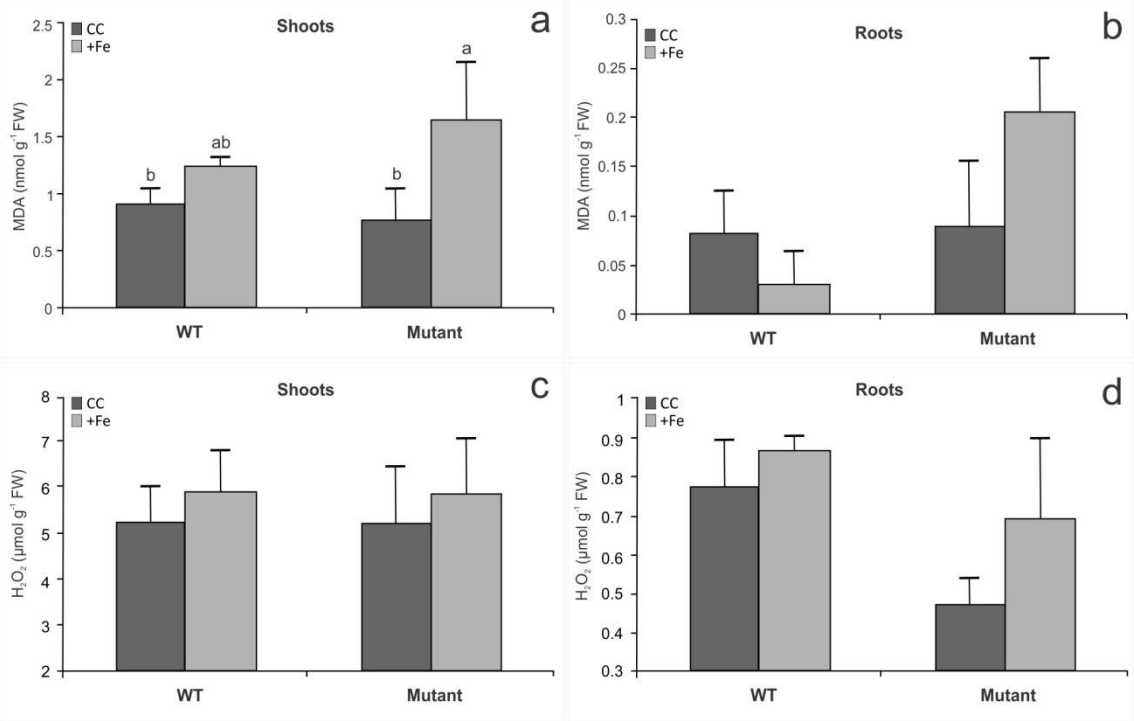

Figure 6: Effect of iron excess in oxidative damage to lipids (MDA concentration) and H_2O_2 concentration. WT and mutant plants were analyzed under control (CC) or iron excess treatment (+Fe). **a** and **b**. MDA concentration, quantified by TBARS. **c** and **d**. Effect of iron stress in hydrogen peroxide content in shoots (c) and roots (d). Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey test ($p \leq 0.05$).

Figure 7

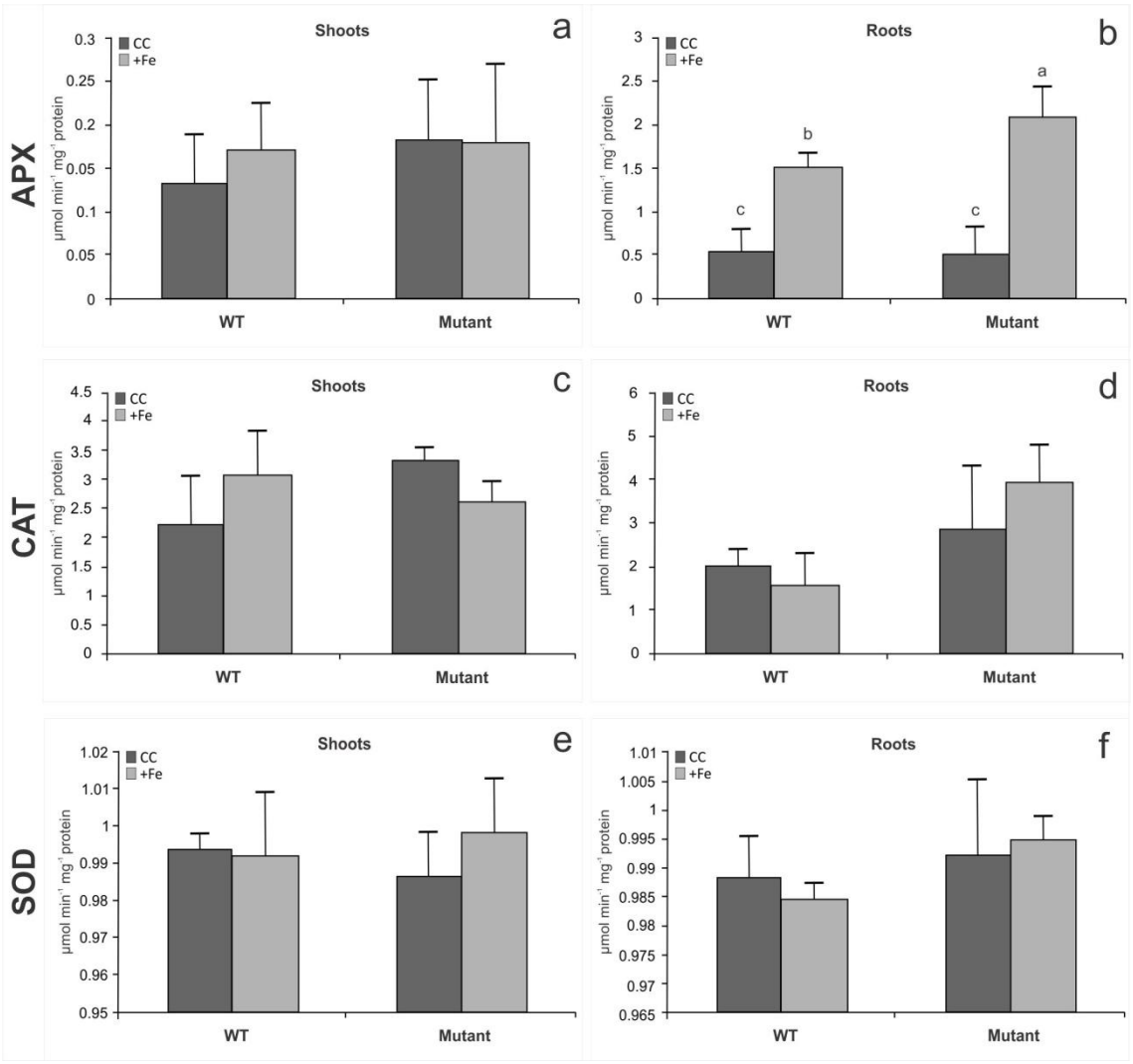

Figure 7: Antioxidant enzyme activity assays. APX (**a**, **b**), CAT (**c, d**) and SOD (**e, f**) activity from shoots and roots of plants subjected to control (CC) or iron excess treatment (+Fe). Values are the averages of ten samples ± SE. Different lower case letters above bars indicate significant difference between means according to the Tukey test ($p \leq 0.05$).

Considerações finais

Sendo o ferro um mineral essencial ao organismo vegetal, vários artigos ressaltam a importância de se aprofundar o conhecimento a cerca de sua homeostase em plantas. Diversos mecanismos envolvidos na homeostase de ferro já foram descritos em *Arabidopsis thaliana*. Contudo, até o momento não temos a mesma quantidade de informação disponível para *Oryza sativa*. Os resultados obtidos sobre a proteína OsNRAMP7 permitiram determinar que a mesma é capaz de mediar o transporte de ferro através de membranas biológicas e, portanto, acredita-se que o mesmo ocorra em células de plantas de arroz. OsNRAMP7 apresenta características específicas da família NRAMP, como o CTM (Consensus Transport Motif) e a sequência conservada DPGN, possivelmente envolvida na ligação de metais à proteína transportadora. A expressão de *OsNRAMP7* levou ao aumento na concentração de ferro em oócitos de *Xenopus*. Portanto, é provável que OsNRAMP7 esteja envolvida na manutenção da homeostase de ferro em plantas de arroz.

Como continuidade a este primeiro trabalho, já estão sendo realizados experimentos de complementação de leveduras deficientes no influxo de metais como ferro e zinco. Mesmo a proteína já tendo sido caracterizada como um transportador transmembrana, experimentos com diferentes linhagens de levedura poderão fornecer indicações sobre a função de OsNRAMP7 como transportadora de membrana plasmática ou tonoplasto. A realização de testes de complementação em leveduras mutantes defectivas na absorção de outros metais, como o zinco, se deve ao fato de a família de transportadores NRAMP estar associada à translocação de outros metais, como manganês, cobalto, zinco, cobre, cádmio, níquel (Ňuňuková et al., 2010) e vanádio (Ueki et al., 2011). A localização subcelular da proteína OsNRAMP7, a ser investigada por meio de sua fusão a GFP e analisada em protoplastos de arroz, deve ajudar a esclarecer se de fato trabalhamos com um transportador vacuolar.

Outras técnicas que possibilitam melhor compreensão do funcionamento da proteína são análises eletrofisiológicas e mutações sítio-dirigidas. Em um projeto aprovado pelo CNPq, a ser realizado em colaboração com o presente grupo, estão previstos tais experimentos. Utilizando a técnica de *voltage-clamp*, correntes elétricas serão medidas na membrana de oócitos injetados com mRNA de OsNRAMP7, determinando assim sua atividade quando submetida a ferro e outros metais. Foram observados no presente trabalho, na estrutura da proteína, motivos possivelmente importantes para a atividade de transporte de

metais pela proteína. Estudos utilizando mutação sítio-dirigida visam investigar a relação entre estrutura e função, bem como a importância de aminoácidos conservados.

A redução na quantidade de ferritina em plantas mutantes *OsFer2* pode estar envolvida em mais do que respostas ao estresse oxidativo gerado por excesso de ferro. Plantas mutantes parecem ter desenvolvimento atípico, caracterizado por plantas menores do que aquelas do tipo selvagem. A ausência de ferritina na parte aérea levou ao aumento na concentração de MDA, subproduto da degradação de lipídios poli-insaturados por espécies reativas de oxigênio. Em raízes de plantas mutantes, o excesso de ferro levou ao aumento da atividade da enzima APX, responsável pela detoxificação de peróxidos na célula. Os resultados obtidos levam a crer que a proteína não é importante para a homeostase de ferro apenas quando a planta se encontra em estresse por excesso do metal, mas também em condições normais de crescimento.

De certa forma, os resultados relativos à proteína OsNRAMP7 podem vir a complementar os estudos com as plantas mutantes para *OsFER2*. Caso a proteína OsNRAMP7 seja mesmo um transportador responsável pelo influxo de ferro para o vacúolo, esta proteína poderia atuar na detoxificação do excesso de ferro em situações de limitação da disponibilidade de ferritina. Melhor compreensão sobre o papel do vacúolo no estresse por excesso de ferro, bem como análises da importância da frataxina, deverão esclarecer os mecanismos utilizados para lidar com situações de estresse. Até o presente momento não existem trabalhos que relatem a função da proteína frataxina em plantas de arroz, apesar de uma cópia ter sido descrita para a espécie (Busi et al., 2004).

Acreditamos que os dados aqui expostos, bem como os demais experimentos que já estão sendo realizados, possam auxiliar na compreensão do intrincado mecanismo relacionado à homeostase de ferro em plantas de arroz.

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