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Gabriel Costa Menezes

Análise do papel de *OsZIFL2* na homeostase de ferro e manganês em plantas de arroz

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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel em Biomedicina.

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RESUMO

A família de transportadores ZIF-like (ZIFL) possui 13 membros em arroz, *OsZIFL-1* a *13*. Alguns membros desse grupo já foram caracterizados como transportadores de moléculas quelantes de metais, como o ácido desoximugineico (DMA) e nicotianamina (NA). Neste trabalho, nós apresentamos um breve estudo com o mutante perda de função para *ZIFL-2* em arroz (*oszifl2*). Primeiro, foi analisado o ionoma de folhas de plantas selvagem (WT, cv. Nipponbare) e mutantes *oszifl2* cultivadas em condições controle. As concentrações de ferro (Fe) e manganês (Mn) são menores nas folhas de plantas *oszifl2*, indicando um possível papel de OsZIFL2 na homeostase desses metais. Após, plantas WT e *oszifl2* foram cultivadas sob quatro condições distintas, controle, deficiência de Fe, deficiência de Mn e excesso de Mn durante 21 dias. Amostras de raízes e folhas foram coletadas para extração de RNA. Também foram realizadas análises morfológicas. Nós quantificamos a expressão de genes relacionados à homeostase de Fe por RT-qPCR. Além disso, parâmetros de crescimento, como comprimento de raízes e parte aérea e número de folhas foram mensurados. Os resultados morfológicos mostram que plantas *oszifl2* apresentam menor comprimento de parte aérea, em comparação com as plantas WT, em situação controle e excesso de Mn. As plantas mutantes também apresentam redução no número de folhas em deficiência de Fe. Análises de RT-qPCR confirmam a regulação de genes associados à estratégia II de captação de Fe em plantas selvagem expostas à deficiência de Fe. Mutantes *oszifl2* sinalizam a deficiência de Fe, através da regulação de *IRO2*, porém não induzem a expressão de genes envolvidos com a captação de Fe, mostrando que *OsZIFL2* têm um papel importante neste processo.

Palavras-chave: Arroz. Homeostase de metais. Manganês. Ferro. ZIF-like.

ABSTRACT

The ZIF-like (ZIFL) family of transporters has 13 members in rice named OsZIFL-1 to 13. Some members are involved in rice metal homeostasis transporting metal-chelating molecules, such as deoxymugineic acid (DMA) and nicotianamine (NA). In this work, we present a brief study with the rice *ZIFL-2* knockout mutant (*oszifl2)*. First, we analyzed the ionome of leaves from wild-type (WT, cv. Nipponbare) and *oszifl2* plants grown in control conditions. Iron (Fe) and manganese (Mn) concentrations are lower in the leaves of *oszifl2* plants, indicating a possible role of *OsZIFL2* transporter in metal homeostasis. Next, WT and *oszifl2* plants were cultivated under control conditions, Fe and Mn deficiency, and Mn excess for 21 days for shoot and root RNA extraction and for morphological analysis. We quantified the expression of rice genes related to Fe homeostasis by RT-qPCR. Also, growth parameters such as shoot and root length, and number of leaves were measured. The morphological results show that *oszifl2* plants have shorter shoot length compared to WT in control conditions and Mn excess. The mutant plants also show a reduction in the number of leaves in Fe deficiency. RT-qPCR analyses confirmed the up-regulation of genes associated with strategy II response to Fe deficiency in WT (Nipponbare) plants. The *oszifl2* mutant signalized Fe deficiency through the regulation of IRO2, although genes involved with Fe uptake were not induced, indicating that OsZIFL2 plays a significant role in this process.

Keywords: Rice. Metal homeostasis. Manganese. Iron. ZIF-like.

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SUMÁRIO

1 INTRODUÇÃO COMPREENSIVA

O arroz é a principal fonte de carboidrato para mais de 50% da população mundial, e em determinadas regiões, como na Ásia, pode representar mais de 70% do consumo calórico para mais de 3 bilhões de pessoas (FAO, 2019). O Brasil é o maior produtor fora do continente asiático (FAO, 2019), com produção de aproximadamente 12 milhões de toneladas durante a safra de 2018/2019 (CONAB, 2019), colocando-o em nono lugar entre os países produtores. No Brasil, o Rio Grande do Sul tem papel central na produção, sendo o maior produtor interno com 69.9% da produção nacional, responsável por aproximadamente 8.5 milhões de toneladas do grão (IRGA, 2019). Sendo uma das três principais culturas do mundo, o arroz (*Oryza sativa*) é também considerado uma planta-modelo em estudos com monocotiledôneas, foi a segunda planta a ter seu genoma sequenciado (YU et al 2002).

O ferro (Fe) e o manganês (Mn) são elementos minerais essenciais para as plantas. O Fe está presente em quatro diferentes estados de oxidação, sob o pH fisiológico, podemos encontrar, ferroso (Fe^{2+}) e férrico (Fe^{3+}), característica que o torna um eficiente doador / aceptor de elétrons. Por sua atividade redox, o Fe possui diversas funções durante o processo de fotossíntese e respiração em cloroplastos e mitocôndrias respectivamente (BRIAT et al. 2010), além de atuar na fixação de nitrogênio, produção de clorofila e síntese de DNA entre tantos outros processos.

Apesar de ser o quarto elemento mais abundante na crosta terrestre, o Fe é pouco disponível para absorção por plantas, devido à sua baixa disponibilidade em pH neutro a básico, nessas condições o Fe possui baixa solubilidade por estar formando óxidos-hidróxidos a partir de Fe^{3+}. Devido a essas características, estima-se que a concentração disponível de Fe em solo esteja entre 10^{-14} e 10^{-17} M, abaixo da concentração ideal para o crescimento de plantas, que se encontra na faixa de 10^{-4} e 10^{-7} M (GUERINOT and YI 1994). Em solos alagadiços, sistema empregado no cultivo de arroz no RS (CONAB, 2019), a concentração de O_2 no solo diminui favorecendo a ação redutora microbiana que reduz Fe^{3+} à Fe^{2+}, que por ser mais solúvel, aumenta sua disponibilidade para absorção da planta. Por estar mais disponível a planta absorve mais Fe do solo, podendo apresentar estado de toxicidade por excesso do mesmo, um dos estresses mais comuns observados em cultivares de arroz (BECKER; ASCH, 2005).

As plantas apresentam duas estratégias para absorver Fe proveniente do solo, a mais presente é denominada estratégia I, ou estratégia de redução, descrita em modelos de Arabidopsis thaliana. Esta estratégia consiste na indução de um conjunto de genes, dentre eles

uma próton ATPase, que faz a extrusão de prótons para a rhizosfera reduzindo seu pH, a fim de aumentar a solubilidade de Fe^{3+}; uma redutase de membrana que reduz Fe^{3+} a Fe^{2+}; e um transportador chamado IRT1 que possui alta afinidade por Fe^{2+} e promove o influxo de Fe para as células radiculares. Já na estratégia II ou de quelação, presente em plantas da família Poaceae, ocorre a síntese e liberação de aminoácidos de baixo peso molecular, o que é feito pelo transportador de efluxo codificado pelo gene *OsZIFL4* em arroz (NOZOYE et al., 2011), que podem ligar-se à diferentes metais, incluindo Fe^{3+}; e no transporte do complexo fitossideróforo-Fe^{3+} para o citoplasma pelo transportador *OsYSL15* (LEE et al., 2009); (INOUE et al., 2009). O arroz por ser pertencente à família Poaceae utiliza a estratégia II para obtenção de Fe, mas diferentemente de outras plantas, foi observado que em situações de deficiência de Fe ocorre a indução de *OsIRT1*. Foi sugerido que o arroz utiliza uma estratégia combinada para absorção de Fe a partir da rizosfera, a qual é composta pela estratégia II completa e, parte da estratégia I, pois o genoma do arroz codifica duas proteínas relacionadas com a estratégia I, IRT1 e IRT2(RICACHENEVSKY and SPEROTTO, 2014).

O Mn é um micronutriente essencial em plantas, age como cofator de enzimas que atuam na fotossíntese, biossíntese de lipídios e possui importante papel na detoxificação de espécies reativas de oxigênio (ROS) via Mn-superóxido dismutase, um sistema antioxidante que converte radicais superóxido em H_2O_2 (NICKELSEN and REGNSTL, 2013). Sua disponibilidade está intimamente relacionada ao pH do solo e à presença de processos de redução-oxidação presentes. Em solos com pH $\leq$ 5.0 a disponibilidade de Mn aumenta, podendo causar toxicidade em plantas, caracterizada por manchas marrons nas folhas (WISSEMEIERet al. 1987) (FUHRS et al. 2009) e diminuição da biomassa e do crescimento (LEIet al. 2007). A capacidade de certas plantas em tolerar o excesso de Mn no solo é altamente variável, cultivares de *Oryza sativa*, que tipicamente são plantados em solos inundados, possuem elevada resistência à toxicidade, visto que suas folhas podem acumular um nível 30x maior de Mn do que a cevada sem apresentar quaisquer sintomas (BARBER et al, 1995).

Dois transportadores envolvidos na aquisição de Mn foram identificados em arroz. *OsNramp5*, que atua como transportador de influxo de Mn da rizosfera para as raízes, *OsMTP8* e *OsMTP9*, que transporta Mn das células radiculares para o estelo (SASAKI et al, 2012 UENO et al, 2015). O arroz possui dois MTPs de grupo 8 (*OsMTP 8* e *OsMTP 8.1*) e três MTPs de grupo 9 (*OsMTP 9, OsMTP 11 OsMTP 11.1*), caracterizados como transportadores de Mn (UENO et al, 2015, CHEN et al, 2013). Também conhecidas como proteínas de tolerância a metais as MTPs são descritas como facilitadores da difusão de

cátions, essas proteínas promovem caminhos alternativos para efluxo de Mn das células através da Membrana plasmática (MIGOCKA et al, 2015), sequestro em vacúolos (Delhaize et al, 2003) ou através do complexo de Golgi (PEDAS et al, 2014). A transcrição de *OsNramp5* e *OsMTP9* não sofre alteração por aumento das concentrações externas de Mn (SASAKI et al, 2012) (UENO et al, 2015), o que poderia indicar uma fraca regulação da captação de Mn (BARBERON et al, 2011). Outro gene proposto como atuante na translocação distal de Mn em plantas é o *OsYSL2*. Sendo primariamente expresso em folhas e localizado em células companheiras do floema é provavelmente envolvido no carregamento de Mn-nicotianamina. Além disso, o aumento da expressão causa maior captação de Mn pelos grãos (ISHIMARU et al, 2010).

Sabendo da importância de elementos como Fe e Mn para o desenvolvimento normal das plantas, torna-se essencial a caracterização dos genes envolvidos na homeostase desses metais. Estudos indicam um grande número de genes que têm papel fundamental no controle da homeostase de elementos como Fe, Mn, dentre outros, nos diversos compartimentos presentes em plantas, muitos dos quais ainda não foram completamente caracterizados. (RICACHENEVSKY et al, 2018).

As proteínas ZIF-like (ZIFL) fazem parte da Major Facilitator Superfamily (MFS). Arroz possui 13 membros, *OsZIFL1-13* e *Arapidopsis* 3, *AtZIF1*, *AtZIFL1* e *AtZIFL2* (Haydon et al, 2007). Alguns dos membros estão envolvidos com a homeostase de metais através do transporte de moléculas que quelam metais, como ácido desoximugineico (DMA) e a NA(HAYDON et al, 2012).Os genes da família *OsZIFL* são alvos promissores para futuros estudos de caracterização, já que fazem parte da regulação da homeostase de metais em plantas de arroz. Caracterizado anteriormente por Haydon e Cobbett em modelos Arabidopsis thaliana o gene *ZIF1* pertencente à mesma família do *OsZIFL2* está claramente envolvido com a homeostase de Zn, já que um mutante com perda de função apresentou alterações na distribuição de Zn na planta e o mesmo se encontra superexpresso em ambientes de excesso de Zn (HAYDON COBBETT et al, 2007). Também já descrevemos aqui a importância de *OsZIFL4* na homeostase de Fe. Neste trabalho mostramos que o mutante perda de função oszifl2 apresenta redução no acúmulo de Fe e Mn na parte aérea; redução em parâmetros morfológicos quando expostos à deficiência de Fe, Mn e excesso de Mn; e também uma regulação diferencial de genes de captação de Fe quando em deficiência desse metal.

1.1 JUSTIFICATIVA

A caracterização de genes relacionados com a homeostase de metais em plantas é fundamental para o desenvolvimento de estratégias de biofortificação por melhoramento ou engenharia genética. A biofortificação busca uma maior qualidade nutricional em partes comestíveis de plantas visando o combate à deficiência de minerais como Fe e Zn em humanos.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Estudar o mutante *oszifl2* em arroz.

1.2.2 Objetivos específicos

1. Analisar o perfil de acúmulo de micronutrientes em folha de plantas WT e *oszifl2*;

2. Analisar parâmetros morfológicos de plantas *oszifl2* expostas à tratamentos de deficiência de Fe, Mn e excesso de Mn;

3. Analisar a expressão de genes envolvidos na homeostase de Fe em raiz de plantas *oszifl2* expostas à deficiência de Fe.

2 ARTIGO CIENTÍFICO

Analysis of *OsZIFL2* role in Iron and Manganese homeostasis in rice.

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Abstract

The ZIF-like (ZIFL) family of transporters has 13 members in rice named OsZIFL-1 to 13. Some members are involved in rice metal homeostasis transporting metal-chelating molecules, such as deoxymugineic acid (DMA) and nicotianamine (NA). In this work, we present a brief study with the rice *ZIFL-2* knockout mutant (*oszifl2)*. First, we analyzed the ionome of leaves from wild-type (WT, cv. Nipponbare) and *oszifl2* plants grown in control conditions. Iron (Fe) and manganese (Mn) concentrations are lower in the leaves of *oszifl2* plants, indicating a possible role of *OsZIFL2* transporter in metal homeostasis. Next, WT and *oszifl2* plants were cultivated under control conditions, Fe and Mn deficiency, and Mn excess for 21 days for shoot and root RNA extraction and for morphological analysis. We quantified the expression of rice genes related to Fe homeostasis by RT-qPCR. Also, growth parameters such as shoot and root length, and number of leaves were measured. The morphological results show that *oszifl2* plants have shorter shoot length compared to WT in control conditions and Mn excess. The mutant plants also show a reduction in the number of leaves in Fe deficiency. RT-qPCR analyses confirmed the up-regulation of genes associated with strategy II response to Fe deficiency in WT (Nipponbare) plants. The *oszifl2* mutant signalized Fe deficiency through the regulation of *IRO2*, although genes involved with Fe uptake were not induced, indicating that *OsZIFL2* plays a significant role in this process.

Keywords: Rice. Metal homeostasis. Manganese. Iron. ZIF-like.

Introduction

Iron (Fe) and Manganese (Mn) are essential mineral elements to plants. Iron is present in four oxidation states under physiologic pH, found in ferrous (Fe^{+2}) and ferric (Fe^{+3}) states, which makes it an efficient electron donor/acceptor. Due to its redox activity, Fe possesses many functions during photosynthesis and respiration (Briat et al. 2010). It also has many roles during nitrogen fixation, chlorophyll production, and DNA synthesis.

Plants developed two strategies to absorb Fe from the soil. The most predominant is called Strategy I, or reduction strategy, described in *Arabidopsis thaliana*. This strategy consists of inducing a set of genes, among them a proton ATPase whose objective is reducing rhizosphere pH in order to increase Fe^{3+} solubility, a membrane reductase which reduces Fe^{+3} to Fe^{+2}; and a transporter called *IRT1* which possesses high affinity to Fe^{+2} promoting Fe influx into root cells. Strategy II, or chelation strategy, present in the Poaceae family, is based on the synthesis and secretion mugineic acids part of the phytosiderophores (PS) family, the last performed by *ZIFL4* in rice (NOZOYE et al., 2011). In the rhizosphere, phytosiderophores bind to different metals, including Fe^{+3}. The PS-Fe^{+3} complex is transported into the root cells by members of the Yellow Stripe family, in rice by *YSL15* (LEE et al., 2009; INOUE et al., 2009). Rice belongs to the Poaceae family and uses strategy II to acquire Fe, but under Fe deficiency conditions, *IRT1* induction occurs. So, it was suggested that rice would utilize both strategies to acquire Fe (Ishimaru et al, 2006).

Manganese participates as an enzyme cofactor in photosynthesis, lipid biosynthesis, and reactive oxygen species (ROS) detoxification (Nickelsen and Rengstl, 2013). Mn levels in soil can suffer high variations due to its close relation to the pH levels. Acidic soil has a higher disponibility of Mn, causing toxic reactions in plants, as shown by brown spots in leaves and biomass reduction (Wissemeier et al. 1987, Fuhrs et al. 2009). The *Oryza* genus is more resistant to Mn toxicity, accumulating 30x more Mn in its leaves without showing signs of toxicity, which is highly beneficial since rice is cultivated in low land, where Mn is widely available (Barber et al, 1995).

The ZIF-like (ZIFL) proteins are all part of the Major Facilitator Superfamily (MFS). Rice has 13 members, *OsZIFL1-13*, and *Arabidopsis* 3, *AtZIF1*, *AtZIFL1* and *AtZIFL2* (Bughio et al, 2002). Some members are involved in metal homeostasis through the transport of metal-chelating molecules, such as deoxymugineic acid (DMA) and nicotianamine (NA) (Haydon, et. al. 2012). *AtZIF1* transporter is a vacuolar membrane protein required for basal Zn tolerance involved in the influx of nicotianamine into the vacuole (Haydon et al., 2012).

As already mentioned, rice *ZIFL4/TOM1* transports phytosiderophores (derivatives of mugineic acid) to the rhizosphere (Nozoye, T. et. al. 2011). In addition, the expression of *OsZIFL4*, *OsZIFL5*, *OsZIFL7*, and *OsZIFL12* is upregulated in response to Zn-excess and Fe-deficiency in roots, two stresses with partially overlapping responses (Ricachenevsky et al., 2011). Arabidopsis ZIFL2 was recently characterized as a K^+ efflux transporter localized in the plasma membrane (Remy et al., 2015). In this work, we show rice mutant plants for *ZIFL2* with a reduction in the accumulation of Fe and Mn in the shoot; reduction in morphological parameters when exposed to Fe deficiency and excess Mn; moreover, a differential regulation of Fe uptake genes when this metal is deficient.

Material and methods

Plant material and treatments

Rice seeds of the Nipponbare cultivar (WT) and *oszifl2* loss-of-function mutant were germinated for four days in petri dishes containing filter paper embedded with water at 28°C (two days in the dark, and two days in the light). Past four days, seedlings were transferred to a grow tray containing individual orifices to accommodate plants in contact with the nutritive solution (1mL/L $FeCl_3$ 10 mM, 1mL/L $CaCl_2$ 0.5 mM, Vitavax) where they were kept for 3 days. Plants were later cultivated in hydroponics at 500 mL plastic recipients with control nutrient solution containing 700 µM K_2SO_4, 100 µM KCl, 100 µM KH_2PO_4, 2 mM $Ca(NO_3)_2$, 500 µM $MgSO_4$, 10 µM H_3BO_3, 0,5 µM $MnSO_4$, 0,5 µM $ZnSO_4$, 0,2 µM $CuSO_4$, 0,01 µM $(NH4)_6Mo_7O_{24}$, e 100 µM Fe(III)-EDTA (Ricachenevsky et al 2011), during seven days for acclimatation, then treated with control solution, -Fe (no Fe added), -Mn (no Mn added) and Mn excess (300µM Mn) for a period of 21 days for growth measurements and RT-qPCR analysis. Growth parameters such as shoot and root length, and number of leaves were measured. Also, plants were kept for 24 days with control nutrient solution for ICP analysis. Past every 3 days the nutrient solutions were changed.

Mineral quantification

Samples from the 3rd expanded leaf were collected when WT and *oszifl2* plants reached five completely expanded leaves (21 days). The element quantification was performed as previously described (Ricachenevsky et al 2018).

Gene expression analyses by RT-qPCR

RNA was extracted from RNA samples using Concert Plant RNA reagent (Invitrogen®, Carlsbad, USA), following the manufacturer instructions. Quantification was done using Nanodrop® (Thermo Fisher Scientific, Waltham, USA). Total RNA was treated with DNAse I (Invitrogen®, Carlsbad, USA) and the first cDNA strand was synthesized using OligodT and M-MLV reverse transcriptase (Invitrogen®, Carlsbad, USA). Final RT-qPCRs volume was adjusted to 20 µL, composed by 10 µL 50x cDNA diluted sample, 2 µL 10x PCR buffer, 1.2 µL 50 mM $MgCl_2$, 0.2 µL 10 mM dNTPs, 0.4 µL of each primer pair (10 µM), 3.82 µL water, 2 µL SYBR green (1:10.000 Molecular Probe) and 0.05 µL of PLatinum Taq DNA Polimerase (5 U μL^{-1}, Invitrogen, Carlsbad, CA, USA). Reactions were performed in a StepOne Real-Time Cycler equipment (Applied Biosystems, Foster City, USA). Reaction parameters were composed by a five minute initial denaturation period at 94°C, followed by forty 10 s cycles at 94°C, 15 s at 72°C then 40 s 60°C, finally data were collected using fluorescence. Samples were kept at 40°C for 2 min so annealing of the amplification products could occur, then heat from 55 to 99°C ascending 0.1°C/s so a denaturation curve of the amplified product would be produced. Expression data analyses were performed after comparative quantification of the amplified products using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008). Primers used are listed in Table 1.

Table 1. Gene-specific PCR primers used for RT-qPCR

Locus	Gene name	Forward Primer 5' → 3'	Reverse Primer 5' → 3'
OS01G0952800	IRO2	CGGATTTGGGAACAGGACA	GTTCCTGACGACTTTCTCCA
OS03G0667500	IRT1	ACTGGTGCCCATTCTGC	GCGAGGATGGGGATGG
OS03G0751100	OPT7	AGTGTGAAGGCGCCG	ATCTTCTTCTTCGCGAGCTT
OS11G0134900	ZIFL4-TOM1	TGTGATTGAATTAATTGGACTTGC	GGGGTGCTATTCCAGCTTCT
OS03G0237100	DMAS	CCTGGACATCGTCGGAT	GTCGTCGAGCGACTTGTAG
OS12G0133100	ZIFL12	CCCAAACTGTTGAAGCTTTGG	GGACATCAAGGGCCAATTTC
OS02G0650300	YSL15	GGTGCGGGGATGATTTG	CCATACAAACTTGTCATGCTG
OS01G0323600	SAMS	ACGCCACCTTGCTGTC	GACGTTCCTCTTCACCTCC
OS01G0328400	UBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

Statistical analysis

When appropriate, data was subject to ANOVA and means were compared by the Tukey HSD or Student's T test, graphical information was then plotted using Prism software 7.0 for windows.

Results

Mineral quantification

The mineral quantification in leaves of WT and *oszifl2* plants grown in control conditions show the mutant with an increase of 30 % for Zn (Fig. 1A) and a decrease of 42 and 50 % for Mn (Fig. 1B) and Fe (Fig. 1C), respectively. These results show an alteration in Zn, Mn, and Fe translocation to the shoot with the loss-of-function of *ZIFL2*. As Mn and Fe have their concentration diminished, we hypothesized that ZIFL2 could have a role in the homeostasis of these two elements in rice plants.

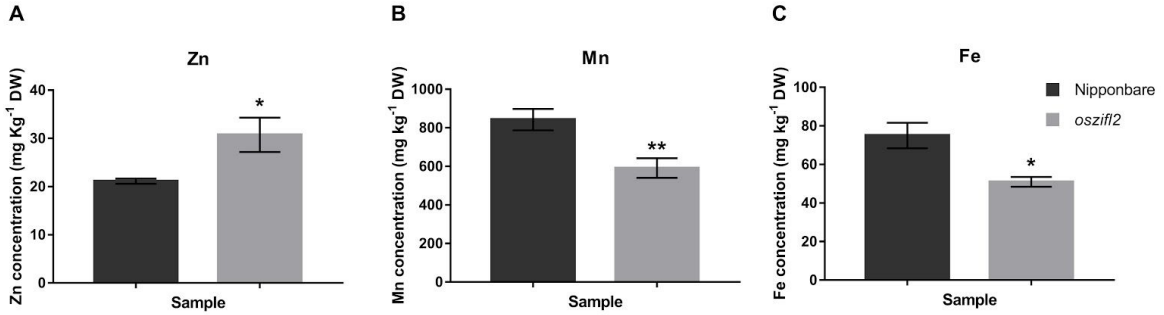

Fig 1. Zinc (Zn), manganese (Mn) and iron (Fe) concentration in leaves of WT cv. Nipponbare and *oszifl2* plants. Values are the averages of six samples ± SE. Statistical differences according to the Student's T-test in comparison to control are shown by one ($p = 0.05$) or two asterisks ($p = 0.01$).

Morphological analysis

We continued the work exposing WT and *oszifl2* plants to control conditions, Fe and Mn deficiency, and Mn excess, to perform some morphological analysis. The *oszifl2* plants showed reduced shoot length compared to WT in control conditions and Mn excess (Fig. 3A). Root length did not show a statistical difference between plants (Fig. 3B). We could observe a difference only in the *oszifl2* plants during Fe deficiency (Fig. 3C) in the leaf count analysis. In a visual examination, no signs of Mn toxicity in the Mn excess treatment were noticed. Iron deficiency treatment resulted in plants with a yellowish color, as expected (Fig. 2A and 2B).

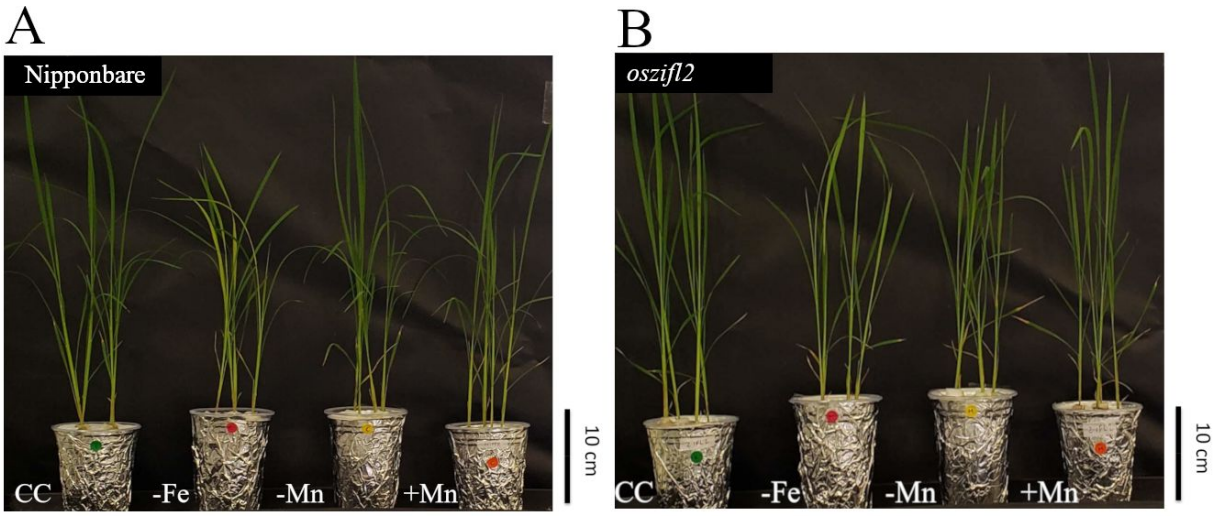

Fig 2. Comparison of WT cv. Nipponbare (A) and *oszifl2* (B) plants exposed to control conditions (cc), iron deficiency (-Fe), manganese deficiency (-Mn), and Mn excess (Mn).

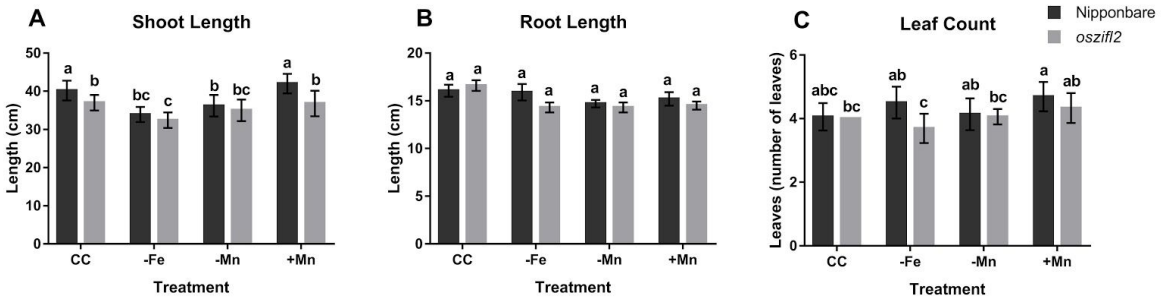

Fig 3. Shoot length (A), root length (B) and number of leaves (c), of WT cv. Nipponbare and *oszifl2* plants exposed to control conditions (cc), iron deficiency (-Fe), manganese deficiency (-Mn), and Mn excess (Mn). Data was subjected to ANOVA and means were compared by the TUKEY HSD using the PRISM software 7.0 for windows.

Expression analysis

We analyzed the expression of eight genes related to iron uptake and homeostasis in WT and *oszifl2* roots of plants exposed to control conditions and Fe deficiency. The *YLS15* gene responsible for iron's direct uptake from the rhizosphere (Murata et al., 2006) is upregulated in Fe deficiency only in WT plants (Fig. 5B). Even though *IRT1* is proposed to be upregulated in Fe deficiency (Ishimaru et al,, 2006), this could not be statistically confirmed in our tests for both WT and *oszifl2* plants (Fig. 5A). *SAMS* is involved in the synthesis of nicotinamine (NA), a critical metal chelator (Inoue et al., 2003). This gene is upregulated in both WT and *oszifl2* plants, the latter in higher magnitude (Fig. 5C). DMAS, an enzyme

accountable for the deoxymugineic acid (DMA) production, has been reported to be up-regulated in Fe deficiency (Bashir et al., 2006). We confirmed this for WT and *oszifl2* plants (Fig. 5D). *IRO2* is a transcription factor (TF) induced in root and shoot of rice plants subjected to Fe deficiency (Ogo et al., 2006). This TF regulates the expression of genes related to Fe^{3+} uptake (Ogo et al., 2007). *IRO2* is upregulated in both WT and *oszifl2* plants under Fe deficiency, the latter in higher magnitude (Fig. 5E). The *OPT7* and *ZIFL4-TOM1* genes, related to Strategy II, are induced in *oszifl2* (Fig. 5F) and WT (Fig. 5G), respectively. *ZIFL12* was not regulated for both plants (Fig. 5H).

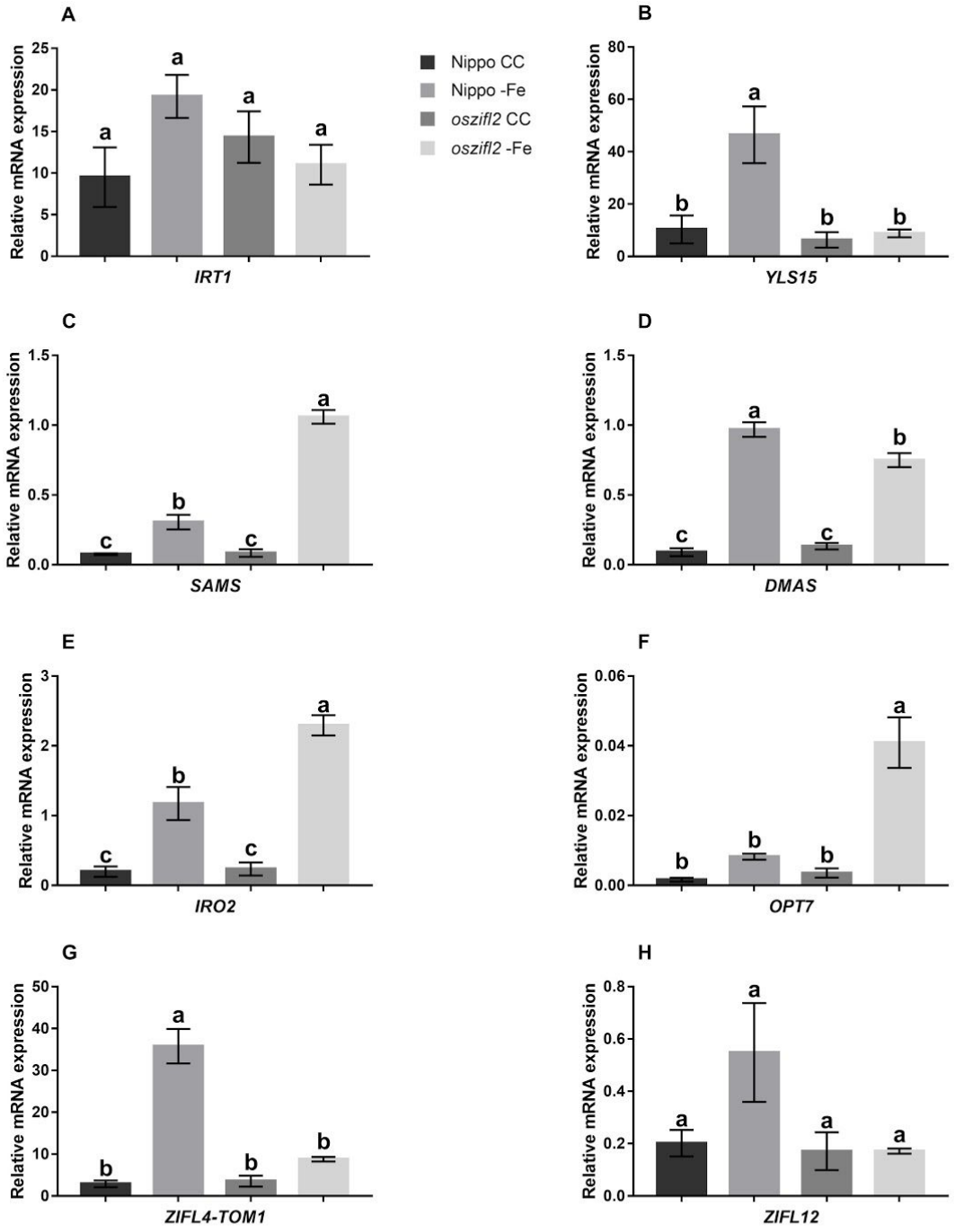

Fig 4. Relative expression of *IRT1* (A), *YSL5* (B), *SAMS* (C), *DMAS* (D), *IRO2* (E), *OPT7* (F), *ZIFL4-TOM1* (G) e *ZIFL12* (H) in WT (Nipponbare) and *oszifl2* plants exposed to control conditions CC and iron deficiency (-Fe). When appropriate, data were subjected to ANOVA and means were compared by the Tukey HSD using the Prism software 7.0 for windows.

Discussion

Previous results show *OsZIFL2* expression upregulated in the root of WT plants exposed to Zn excess; this gene is also responsive to arsenate. Moreover, iron deficiency does not regulate the expression of *OsZIFL2* in both root and leaves (Ricachevesky et al., 2011). This work revealed that *oszifl2* loss-of-function mutant shows reduced Fe and Mn concentrations in leaves under control conditions (Fig. 1). The same happens under Zn, Fe, and Mn excess (data not shown). In this way, *oszifl2* presents a consistent alteration in Fe and Mn homeostasis among treatments. The *oszifl2* plants exposed to Fe and Mn deficiency, and Mn excess, has reduced shoot length in Mn excess (Fig. 3A), and less number of leaves under Fe deficiency (Fig. 3C). These results suggest that ZIFL2 is involved in Mn and Fe homeostasis in rice plants.

We conducted an experiment to analyze genes related to Fe uptake and homeostasis in WT and *oszifl2* root of plants exposed to Fe deficiency. Iron uptake via Strategy II was signaled by *IRO2* in both plants under Fe deficiency, however, *oszifl2* showed an upregulation of 2 times in comparison to WT (Fig. 4E). We suggest that *oszifl2* is responding to a most severe Fe deficiency, which correlates with the lower Fe concentration found in these plants. Interestingly, *YSL15* and *ZIFL4-TOM1* involved in Strategy II, and induced via IRO2 (Ogo et al., 2007), are not regulated in *oszifl2* (Fig. 4B and G). The upregulation of these two genes is essential for Fe^{3+} uptake by the roots under Fe deficiency (Nozoye et al., 2011) (Lee et al., 2009) (Inoue et al., 2009). The lack of induction in *oszifl2* shows the importance of ZIFL2 in Strategy II Fe^{3+} uptake. The *ZIFL* genes are known to be part of metal homeostasis in plants, several members are involved in the transport of metal-chelating molecules like DMA and NA (Haydon, et. al. 2012). *DMAS* and *SAMS* expressions were upregulated in both plants, the latter three times more in *oszifl2* than WT (Fig 4C and D). The difference in *SAMS* regulation between *oszifl2* and WT plants under Fe deficiency could indicate the involvement of OsZIFL2 in the transport of metal chelators.

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3 CONCLUSÕES E PERSPECTIVAS

Diante dos resultados obtidos, pudemos inferir que ZIFL2 está envolvido na homeostase de Fe e Mn em plantas de arroz. Na resposta à deficiência de Fe, ele apresenta papel fundamental para captação de Fe^{+3}. Como principais perspectivas está a análise da expressão de *ZIFL2* em plantas de arroz WT expostas à deficiência de Mn e excesso de Mn, como também analisar a expressão de genes relacionados com a homeostase e captação de Mn, tanto em plantas WT quanto em *oszifl2* submetidos aos mesmos tratamentos.

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ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA PHYSIOLOGY AND MOLECULAR BIOLOGY OF PLANTS

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