Universidade Federal do Rio Grande do Sul

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Caracterização funcional dos genes *OsZIP3*, *OsFRO1* e *OsFRO2* de arroz (*Oryza sativa* L.)

Dissertação submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular da UFRGS como requisito parcial para a obtenção do grau de Mestre.

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"The truth may be puzzling. It may take some work to grapple with. It may be counterintuitive. It may contradict deeply held prejudices. It may not be consonant with what we desperately want to be true. But our preferences do not determine what's true."

Carl Sagan

"There are numerous 'styles' for doing science: the only constant is the need to measure one's ideas against the real world."

Lewis Wolpert

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Índice

Lista de Abreviaturas

Ah = *Arachis hypogaea* [amendoim]

AHA = *Arabidopsis* H^+-pump ATPase [ATPase bomba de H^+ de *Arabidopsis*]

At = *Arabidopsis thaliana* [sem nome popular]

ATP = adenosine triphosphate [trifosfato de adenosina]

cDNA = complementary DNA [DNA complementar]

Cs = *Cucumis sativus* [pepino]

DMAS = deoxymugineic acid synthase [ácido deoximugineico sintase]

DNA = desoxyribonucleic acid [ácido desoxirribonucleico]

DW = dry weight [peso seco]

EDTA = ethylenediamine tetracetic acid [ácido etilenodiamino tetra-acético]

epiHDMA = 3-epihydroxy-2'-deoxymugineic acid [ácido 3-epihidroxi-2'-deoximugineico]

epiHMA = 3-epihydroxymugineic acid [ácido 3-epihidroximugineico]

EST = expressed sequence tag [etiqueta de seqüência expressa]

FAD = flavin adenine dinucleotide [flavina adenina dinucleotídeo]

FER = ferritin [ferritina]

FPN = ferroportin [ferroportina]

FRD = ferric reductase defective [defectivo em redutase férrica]

FRO = ferric reductase oxidase [oxidase redutase férrica]

HMA = heavy metal-associated domain proteins [proteínas com domínio associado a metal pesado]

Hs = *Homo sapiens* [humano]

Hv = *Hordeum vulgare* [cevada]

IDE = iron deficiency-responsive element [elemento responsivo à deficiência de ferro]

IDEF = IDE factor [fator de IDE]

IDRS = iron-dependent regulatory sequence [sequência regulatória dependente de ferro]

IDS = iron deficiency-specific clone [clone específico de deficiência de ferro]

IRT = iron-regulated tranporter [transportador regulado por ferro]

LZT = LIV-1 subfamily of ZIP zinc transporters [subfamília LIV-1 de transportadores ZIP de zinco]

MA = mugineic acid [ácido mugineico]

mRNA = messenger RNA [RNA mensageiro]

MTP = metal tolerance protein [proteína de tolerância a metal]

NA = nicotianamine [nicotianamine]

NAAT = nicotianamine aminotransferase [nicotianamina aminotransferase]

NADPH = nicotianamide adenine dinucleotide phosphate [nicotinamida adenina dinucleotídeo fosfato]

NAS = nicotianamine synthase [nicotianamina sintase]

NRAMP = natural resistance-associated macrophage protein [proteína de resistência natural associada a macrófago]

Os = *Oryza sativa* [arroz]

PCR = polymerase chain reaction [reação em cadeia da polimerase]

Ps = *Pisum sativum* [ervilha]

PS = phytosiderophore [fitosideróforo]

qPCR = quantitative PCR [PCR quantitativo]

RNA = ribonucleic acid [ácido ribonucleico]

RT = recombination tag [etiqueta de recombinação]

SAM = S-adenosyl-methionine [S-adenosil metionina]

SAMS = S-adenosyl-methionine synthetase [S-adenosil-metionina sintetase]

Sc = *Saccharomyces cerevisiae* [levedura]

SE = standard error [erro padrão]

Sl = *Solanum lycopersicum* [tomate]

TMD = transmembrane domain [domínio transmembrana]

YSL = yellow stripe like protein [proteína do tipo *yellow stripe*]

ZIF = zinc-induced facilitator [facilitador induzido por zinco]

ZIP = zinc-regulated, iron-regulated-like protein [proteína do tipo zinco-regulada, ferro-regulada]

Zm = *Zea mays* [milho]

Resumo

As plantas necessitam de um conjunto de elementos químicos essenciais, entre eles os metais ferro, zinco, manganês, níquel e cobre. Contudo, esses metais podem se tornar tóxicos quando presentes em excesso dentro das células. Desse modo, as plantas possuem mecanismos fisiológicos para manter níveis apropriados desses elementos. O ferro e o zinco estão entre as três maiores deficiências nutricionais em humanos ao redor do mundo, e um maior conhecimento sobre os mecanismos que regulam sua homeostase poderá auxiliar em intervenções para produção de linhagens com maior conteúdo de metais em suas partes comestíveis. O arroz é um dos cereais mais cultivados e consumidos no mundo, e diversas famílias gênicas possivelmente relacionadas com a homeostase de ferro e zinco foram identificadas no genoma dessa planta. Entre elas, estão a família ZIP de transportadores de metais e a família FRO de redutases férricas. Este estudo procurou caracterizar funcionalmente os genes *OsZIP3*, *OsZIP4*, *OsZIP7* e *OsFRO1* através da análise de mutantes por inserção do retrotransposon *Tos17*. Os mutantes *zip4* e *zip7* não apresentaram alterações fenotípicas drásticas quando comparados ao tipo selvagem. Já o mutante *zip3* apresentou um fenótipo distinto durante os estágios reprodutivos: maior número de panículas e menor produção de sementes. Essa menor produção de sementes se deve a uma maior quantidade de espiguetas vazias, a qual possivelmente é causada por uma menor viabilidade do pólen. De fato, grãos de pólen de plantas *zip3* apresentaram metade da concentração de Zn observada no tipo selvagem. Esses dados sugerem um papel no carregamento de Zn para os grãos de pólen para *OsZIP3*. Uma análise da sequência de diversos genes FRO de angiospermas indicaram que essa família é relativamente bem conservada e que pode ser subdividida em três grupos. Aparentemente, dicotiledôneas possuem mais ortólogos FRO em seus genomas do que monocotiledôneas. A maioria dos genes FRO de monocotiledôneas agrupou-se dentro da subfamília 2, que parece ser constituída por genes de arquitetura conservada e expressos em partes aéreas. Desse modo, resolvemos caracterizar funcionalmente *OsFRO1* e *OsFRO2* de arroz por expressão heteróloga em leveduras para determinar sua atividade de redutase, e a caracterização fenotípica de dois mutantes *fro1* por inserção do retrotransposon *Tos17*.

Abstract

Plants require a set of essential chemical elements, among them the metals iron, zinc, manganese, nickel and copper. However, these metals might become toxic when present in excess inside cells. Thus, plants possess physiological mechanisms to maintain appropriate levels of these elements. Iron and zinc deficiencies are among the three major nutritional limitations in humans worldwide, and a wider knowledge about their homeostasis in plants might help interventions to generate lineages with higher metal contents in their edible parts. Rice is one of the most cultivated and consumed crops around the world, and many gene families possibly related to iron and zinc homeostasis were identified in the genome of this plant. Among them are the ZIP family of metal transporters and the FRO family of ferric reductases. This study aimed to functionally characterize the *OsZIP3*, *OsZIP4*, *OsZIP7* and *OsFRO1* genes by the analysis of *Tos17* insertion loss-of-function mutants. The *zip4* e *zip7* mutants did not present any drastic phenotypic alterations when compared to wild type. In contrast, the *zip3* mutant presented a distinct phenotype during reproductive stages: higher number of panicles and reduced seed yield. This lower seed yield was caused by the increased number of empty spikelets, which is possibly the result of lower pollen viability. In fact, pollen grains from *zip3* plants presented half of the Zn concentration found in wild type. These data suggest a role in Zn loading to pollen grains for *OsZIP3*. Analysis of several genes FRO genes from flowering plants indicated that this family is relatively well conserved and that it can be subdivided in three groups. Apparently, dicots possess more FRO orthologs in their genomes than monocots. The majority of monocot FRO genes clustered inside subfamily 2, which seems to comprehend genes of conserved architecture and expressed in shoots. Therefore, we decided to functionally characterize rice *OsFRO1* and *OsFRO2* by heterologous expression in yeast to determine their reductase activity, and phenotypic characterization of two *fro1* mutants derived from insertion of the retrotransposon *Tos17*.

Introdução

As plantas necessitam de um conjunto de elementos químicos essenciais (nutrientes), sem os quais elas são incapazes de completar o seu ciclo de vida (Figura 1; Barker & Pilbeam, 2007). Esses elementos são obtidos principalmente a partir do solo, à exceção do carbono, do oxigênio e do hidrogênio, que são obtidos a partir do ar ou da água (o nitrogênio também pode ser obtido a partir do ar naquelas espécies de plantas que mantêm relações simbióticas com bactérias fixadoras de nitrogênio; Postgate, 1998). Os outros elementos são tradicionalmente classificados em macro e micronutrientes, dependendo da quantidade exigida para o desenvolvimento normal da planta (Tabela 1; Marschner, 1995). Nesse contexto, as plantas enfrentam a necessidade de capturar e translocar quantidades suficientes desses nutrientes sob condições de grande variação na composição do solo ao longo do tempo (Krämer et al., 2007). Dentre os micronutrientes, encontram-se os metais de transição (nos quais o elétron de maior energia está no orbital d) manganês (Mn), ferro (Fe), níquel (Ni), cobre (Cu) e zinco (Zn). Os metais de transição implicam em um desafio particular às plantas devido à estreita faixa de concentrações fisiologicamente adequadas que existe entre a deficiência e a toxicidade. Isso porque, quando presentes em excesso dentro das células, esses metais se tornam prejudiciais ao funcionamento do metabolismo vegetal. A fim de manter as concentrações desses metais dentro dos limites apropriados e lidar com os efeitos tóxicos da sobrecarga desses elementos, as plantas evoluíram mecanismos controlados e sofisticados de homeostase (i.e., a propriedade de um sistema em regular o seu ambiente interno e manter uma condição estável e constante), os quais garantem o balanço correto entre os processos de captura, quelação, distribuição e armazenamento (Puig & Peñarrubia, 2009).

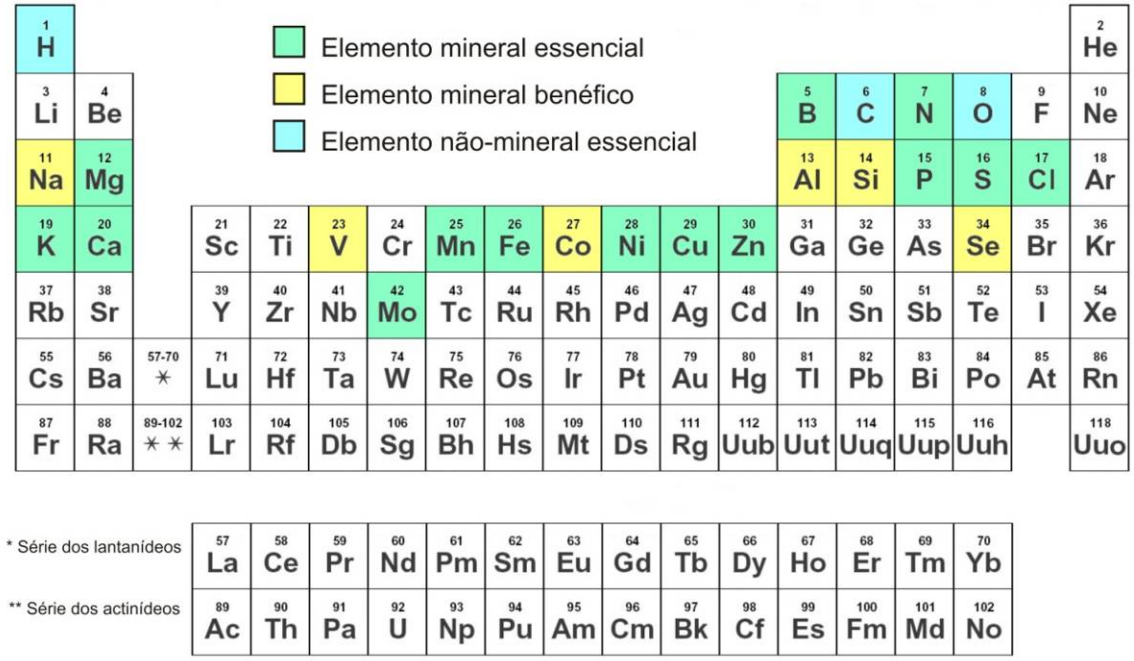


Figura 1. Elementos essenciais e benéficos em plantas vasculares (conforme Barker & Pilbeam, 2007). O hidrogênio, o carbono e o oxigênio são considerados elementos não-minerais, pois são obtidos a partir da água e do ar (em azul). Os outros elementos são considerados minerais e devem ser obtidos a partir do solo ou de soluções nutritivas. Dentre estes, quatorze elementos são considerados essenciais (sem os quais as plantas não completam seu ciclo de vida; em verde) e seis são considerados benéficos (estimulam o crescimento vegetal ou são exigidos somente por algumas espécies de planta; em amarelo).

Tabela 1. Concentrações típicas suficientes para o crescimento vegetal (Epstein, 1965).

Elemento	Símbolo	mg kg^{-1}*	Porcentagem*	Número relativo†
Nitrogênio	N	15.000	1,5	1.000.000
Potássio	K	10.000	1,0	250.000
Cálcio	Ca	5.000	0,5	125.000
Magnésio	Mg	2.000	0,2	80.000
Fósforo	P	2.000	0,2	60.000
Enxofre	S	1.000	0,1	30.000
Cloreto	Cl	100	-	3.000
Ferro	Fe	100	-	2.000
Boro	B	20	-	2.000
Manganês	Mn	50	-	1.000
Zinco	Zn	20	-	300
Cobre	Cu	6	-	100
Molibdênio	Mo	0,1	-	1
Níquel	Ni	0,1	-	1

*As concentrações e porcentagens são relativas ao peso seco.

†Número relativo de átomos estimado após arbitrar um átomo para os elementos encontrados em menor quantidade.

A importância desses metais fica evidenciada pelo fato de que as metaloproteínas constituem um terço de todas as proteínas estruturalmente caracterizadas com atividade

biológica (Finney & O'Halloran, 2003). A existência intercambiável de diferentes formas iônicas (e.g., Cu^{+} e Cu^{2+}) e a afinidade por grupos funcionais que ocorrem em proteínas são propriedades únicas dos metais de transição, o que os tornam úteis em reações bioquímicas redox (Hanikenne et al., 2008). Contudo, essas mesmas propriedades químicas que tornam os íons metálicos bons cofatores também são responsáveis por reações indesejadas no ambiente celular. Justamente porque eles conseguem estabelecer e manter diversas ligações coordenadas estáveis com átomos doadores de ligantes orgânicos, os metais de transição na sua forma iônica podem se tornar tóxicos quando suas interações com ligantes não são totalmente controladas. Reações redox descontroladas, induzidas por metais, ou o deslocamento de cofatores metálicos endógenos de seus sítios celulares de ligação podem levar à toxicidade celular e comprometer a sobrevivência do organismo (Stohs & Bagchi, 1995; Goyer, 1997). As duas seções subseqüentes irão detalhar alguns aspectos dos dois metais de transição mais abundantes em organismos vivos: o Fe e o Zn.

Ferro

O Fe é o metal de transição de número atômico 26, sendo o metal de transição mais abundante nos organismos vivos (Tabela 1). O Fe apresenta duas características químicas que explicam em grande parte a sua presença numa ampla gama de sítios ativos de metaloproteínas (Barker & Pilbeam, 2007). Primeiro, o Fe é capaz de formar seis ligações coordenadas com átomos doadores de elétrons (tais como o oxigênio, o nitrogênio e o enxofre), o que permite que ele se ligue a proteínas sob a forma de grupos heme, *clusters* Fe-S e também como Fe não-heme. Segundo, o Fe existe em dois estados de oxidação sob pH fisiológico – os íons ferroso (Fe^{2+}) e férrico (Fe^{3+}) – o que o torna um eficiente doador e aceptor de elétrons. Devido a essas características, o Fe exerce uma função crítica na maioria

das reações redox necessárias para a produção (fotossíntese) e consumo (respiração) de oxigênio por sua presença em muitas proteínas das cadeias transportadoras de elétrons plastídicas e mitocondriais (Briat et al., 2007). Além disso, o Fe também está envolvido em outros processos metabólicos essenciais, tais como a síntese da clorofila, a fixação do nitrogênio e a síntese de DNA. Algumas enzimas que contêm Fe em plantas são os citocromos, a catalase, as peroxidases, a ferredoxina, a ferro-superóxido dismutase, a aconitase e as lipoxigenases (Marschner, 1995).

Plantas crescidas sob suprimento de Fe insuficiente (i.e., deficiência de Fe) têm o seu desenvolvimento prejudicado e apresentam um conjunto de sintomas, dentre os quais os mais facilmente detectáveis são a inibição do crescimento e a clorose (deficiência de clorofila) nas folhas jovens (Barker & Pilbeam, 2007). Considerando que a maior parte do Fe no interior das células vegetais está localizada no interior do cloroplasto (Terry & Low, 1982), não é de surpreender que essa seja a organela mais afetada pela deficiência desse metal: a clorose ocorre de modo concomitante com um decréscimo nas taxas fotossintéticas e mudanças na estrutura dos cloroplastos (Spiller & Terry, 1980), além da diminuição na expressão da rubisco, de proteínas que se ligam à clorofila e de enzimas envolvidas na síntese de clorofila (Spiller et al., 1987; Winder & Nishio, 1995; Belkhodja et al., 1998). A deficiência de Fe também altera a composição de lipídios e proteínas do tilacóide (Nishio et al., 1985); reduz a capacidade de transporte de elétrons no tilacóide (Spiller & Terry, 1980); e diminui os níveis de ATP nas folhas (Arulanantham et al., 1990). Todos esses efeitos são observados com maior intensidade nas folhas jovens, devido à baixa mobilidade desse elemento através do floema (Marschner, 1995). As raízes também sofrem alterações sob deficiência de Fe, tais como inibição do alongamento, aumento no diâmetro da zona apical e abundante formação de pêlos radiculares (Römheld e Marschner, 1981; Chaney et al. 1992).

Por outro lado, o Fe em excesso dentro das células é capaz de atuar como uma potente toxina quando não está apropriadamente isolado de biomoléculas suscetíveis (Hell & Stephan, 2003). Muitas reações intracelulares usam o oxigênio molecular como aceptor de elétrons, produzindo ânions superóxido (O_2^-) e peróxido de hidrogênio (H_2O_2), os quais não são prejudiciais *per se*, mas contribuem para a geração do extremamente reativo radical hidroxila ($OH^{\bullet}$). A formação desse radical é catalisada pelo Fe através da reação de Haber-Weiss (Equação 3), que é a soma da redução do Fe^{3+} pelo ânion superóxido (Equação 1) e a reação de Fenton (Equação 2; Briat, 2002).

$$(1)\ Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$(2)\ Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$$

$$(3)\ O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

A produção desses radicais pelo Fe livre e também por alguns quelatos de Fe de baixa massa molecular é responsável por gerar estresse oxidativo e conseqüente dano celular (Halliwell & Gutteridge, 1984). Os principais sintomas de toxidez por excesso de Fe são o bronzeamento das folhas (inicialmente as mais velhas) e subseqüente deposição de pigmentos marrons, podendo levar a retardo no crescimento, baixa produtividade, esterilidade, e em casos mais severos, morte da planta (Ponnamperuma et al., 1955). Logo, tanto a manutenção dos níveis intracelulares de Fe quanto o armazenamento do Fe em formas não-tóxicas (e.g., ligado a quelantes orgânicos) devem ser processos finamente controlados (Morrissey & Guerinot, 2009).

Zinco

O Zn é o metal de número atômico 30, sendo o segundo metal de transição mais abundante em organismos vivos. Apesar de ser classificado como um metal de transição por alguns autores, o Zn pode ser considerado um metal de pós-transição, pois seu subnível d é completo (assim como o cádmio e o mercúrio; Jensen, 2003). Por essa razão, ele apresenta propriedades químicas distintas de outros metais de transição, tais como sua existência somente no estado de oxidação +2 (Barak & Helmke, 1993). Portanto, em contraste com o Fe, o Zn não participa diretamente de reações redox (Marschner, 1995). O Zn tem uma forte tendência em formar complexos tetraédricos com átomos doadores de elétrons (nitrogênio, oxigênio e especialmente enxofre), e por isso é amplamente encontrado como um componente integral da estrutura de proteínas. De fato, o Zn é o único metal representado em todas as seis classes de enzimas (Broadley et al., 2007), e mais de setenta metaloproteínas contendo Zn já foram identificadas (Barak & Helmke, 1993), entre elas: a álcool desidrogenase, a anidrase carbônica, a cobre-zinco-superóxido dismutase, a fosfatase alcalina, a fosfolipase, a carboxipeptidase e a RNA polimerase (Marschner, 1995).

Esse papel do Zn como componente estrutural de diversas enzimas o torna um elemento crucial para diversos processos metabólicos nas plantas, tais como a fotossíntese, a formação de sacarose e amido, a síntese protéica, a manutenção da integridade da membrana, o metabolismo de auxina e a reprodução (Marschner, 1995; Barker & Pilbeam, 2007). Uma das funções mais conspícuas do Zn está relacionada com a regulação da expressão gênica: a maior classe de proteínas ligantes de Zn é a daquelas que contêm o domínio *zinc-finger*. Essas proteínas dependem da presença do Zn para reconhecer seqüências de DNA específicas e ativar a transcrição de genes (Alberts et al., 1998; Brown, 2006).

Quando o suprimento de Zn disponível para a planta é inadequado (i.e., deficiência de Zn), uma ou mais das importantes funções fisiológicas do Zn é incapaz de operar normalmente e o crescimento da planta é prejudicado (Alloway, 2004). As mudanças no metabolismo causadas pela deficiência de Zn são complexas, mas algumas dessas mudanças são típicas e podem ser relativamente bem explicadas pelas funções do Zn em reações enzimáticas específicas (Marschner, 1995). Os sintomas característicos mais visíveis da deficiência de Zn são: crescimento reduzido e folhas cloróticas, necróticas e/ou mal formadas. Várias mudanças bioquímicas também podem ser relacionadas à deficiência de Zn: redução da atividade fotossintética, produção de radicais livres, diminuição da síntese protéica, redução dos níveis do fitormônio ácido indol-acético, entre outras (Marschner, 1995).

Entretanto, o excesso de Zn também é prejudicial para as plantas, levando a sintomas como inibição do alongamento radicular e clorose nas folhas jovens (Marschner, 1995). O mecanismo molecular de toxicidade do Zn ainda não está claramente elucidado, mas sabe-se que íons metálicos se ligam a compostos orgânicos, tais como os sítios de ligação a metal de apometaloproteínas, com diferentes afinidades (Nieboer & Richardson 1980; Fraústo da Silva & Williams 2001). Conforme a série de Irving-Williams, primeiro o Cu e depois o Zn são os metais com maior afinidade a esses sítios (Irving & Williams, 1953). Portanto, o Zn seria capaz de competir pelos sítios de ligação de proteínas que contenham metais de menor afinidade, em particular o Fe^{2+} e o Mg^{2+}, que apresentam raio iônico similar ao do Zn^{2+}; e isso levaria a uma deficiência induzida desses outros metais. De modo semelhante ao Fe, a disponibilidade de Zn dentro das células deve ser estritamente regulada para evitar os efeitos nocivos decorrentes de níveis muito elevados ou muito reduzidos.

Transportadores de metais pesados

Progressos consideráveis vêm sendo alcançados na compreensão da homeostase de metais em plantas. O seqüenciamento completo dos genomas de plantas-modelo como a dicotiledônea *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000) e a monocotiledônea arroz (*Oryza sativa* L.; Goff et al., 2002) tem permitido a identificação de famílias gênicas completas envolvidas em diferentes aspectos da homeostase de metais, incluindo transportadores, fatores de transcrição, enzimas e proteínas de armazenamento (Pilon et al., 2009; Puig & Peñarrubia, 2009).

Sabe-se que todos os íons metálicos – incluindo o Fe e o Zn – são incapazes de atravessar membranas lipídicas por difusão passiva, e a ação de proteínas é necessária em cada etapa de transporte transmembrana ao longo do corpo da planta: (1) absorção a partir do solo; (2) entrada e saída de compartimentos apoplásticos, como o xilema; e (3) transporte simplástico através de membranas organelares. Em conformidade com esse fato, um grande número de famílias gênicas envolvidas no transporte de metais pesados vem sendo identificado, entre elas: Zinc-regulated, Iron-regulated-like Protein (ZIP; Guerinot, 2000), Zinc-Induced Facilitator (ZIF; Haydon & Cobbett, 2007a), Metal Tolerance Protein (MTP; van der Zaal, 1999), Natural Resistance-Associated Macrophage Protein (NRAMP; Curie et al., 2000), Yellow Stripe Like (YSL; Curie et al., 2009), Heavy Metal-Associated domain proteins (HMA; Hussain et al., 2004) e as ferroportinas (FPN; Morrissey et al., 2009).

O papel individual dos genes e proteínas pertencentes a essas famílias está começando a ser elucidado, mas a contribuição exata de cada membro na captura, na distribuição para organelas e no armazenamento de metais essenciais, assim como na detoxificação de metais não-essenciais, ainda está por ser decifrada (Hanikenne et al., 2008). De qualquer maneira, um tema recorrente nos estudos já realizados sobre esses transportadores é a grande amplitude

no espectro de substratos; ou seja, uma mesma proteína é capaz de mediar o transporte de mais de um metal essencial e de metais não-essenciais também (por exemplo, o cádmio), mesmo que com diferentes afinidades (e.g., AtIRT1, ver a seguir).

A regulação dos níveis de diferentes metais acaba se sobrepondo devido a essa amplitude de substratos dos transportadores envolvidos, e as plantas devem ter mecanismos para lidar com solos e soluções nutritivas que apresentem desequilíbrios no suprimento de nutrientes. Por exemplo, o fenômeno da deficiência de um metal levar ao excesso de outros já foi observado em diversas espécies. Plantas de cevada (*Hordeum vulgare* L.) sob deficiência de Mn apresentaram concentrações elevadas de Fe, Zn e Cu; enquanto que as deficiências de Fe e Cu levaram a maiores concentrações de Mn e Zn (Pedas et al., 2008). A deficiência de Zn resultou em concentrações maiores de Fe nas raízes de arroz (Ishimaru et al., 2005), e a deficiência de Fe levou ao excesso de Zn em plantas de milho (*Zea mays* L., Kanai et al., 2009).

De fato, quando sob deficiência ou excesso de algum metal, as plantas mobilizam um conjunto de processos fisiológicos com a finalidade de restabelecer as concentrações celulares apropriadas do metal em questão, e um dos casos mais bem caracterizados a nível molecular desses conjuntos de processos são as respostas à deficiência de Fe induzidas na raiz.

Captação de ferro pelas plantas

Em condições de suficiência de Fe, as plantas reduzem quelatos de Fe e transportam o Fe resultante através da membrana plasmática através de um transportador de baixa afinidade, ainda não caracterizado em nível molecular (Curie & Briat, 2003). Em condições de

deficiência, duas estratégias distintas para uma maior captura de Fe evoluíram nas plantas vasculares.

A **estratégia I** é utilizada por dicotiledôneas e monocotiledôneas não-gramíneas e consiste em três processos coordenadamente induzidos nas raízes (Figura 2; Palmer & Guerinot, 2009): (1) extrusão de prótons por bombas dependentes de ATP para acidificar a rizosfera e aumentar a solubilidade do Fe^{3+} (e.g., AtAHA2, Arabidopsis H^+-pump ATPase; Santi et al., 2009); (2) redução do Fe^{3+} por redutases férricas ligadas à membrana (e.g., AtFRO2, Ferric Reductase Oxidase; Robinson et al., 1999); e (3) absorção do Fe^{2+} por transportadores transmembrana de Fe (e.g., AtIRT1, Iron-Regulated Transporter; Eide et al., 1996). A proteína AtIRT1 de *Arabidopsis*, pertencente à família ZIP, é um bom exemplo da amplitude de substratos citada anteriormente, pois foi demonstrado por diferentes métodos que essa proteína não transporta apenas Fe^{2+}, mas também Mn^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+} e Ni^{2+} (Eide et al., 1996; Korshunova et al., 1999; Connolly et al., 2002; Vert et al., 2002; Schaaf et al., 2006). De fato, a análise do perfil metálico de plantas de *Arabidopsis* crescidas sob diferentes concentrações de Fe indica que o crescimento da parte aérea é reduzido pela deficiência de Fe, contudo Zn, Mn, Co e Cd continuam sendo absorvidos por AtIRT1 e a concentração desses metais acaba por aumentar na parte aérea (Baxter et al., 2007).

A **estratégia II** é utilizada por gramíneas e envolve a liberação de compostos de baixa massa molecular na rizosfera conhecidos como fitosideróforos (PS), os quais são derivados estruturais do ácido mugineico e apresentam uma alta afinidade por Fe^{3+} (Figura 2 e Figura 3). Todos os genes envolvidos na síntese de PS foram identificados e caracterizados primeiramente em cevada e sua expressão é induzida em raízes sob deficiência de Fe (Figura 3): S-adenosil-metionina sintetase (SAMS; Takizawa et al., 1996); nicotianamina sintase (NAS; Higuchi et al., 1999); nicotianamina aminotransferase (NAAT; Takahashi et al., 1999);

ácido deoximugineico sintase (DMAS; Bashir et al., 2006) e as dioxigenases IDS2 (Okumura et al., 1994; Nakanishi et al., 2000) e IDS3 (Nakanishi et al., 1993; 2000; Iron Deficiency-Specific Clone). Alguns desses genes também foram caracterizados em arroz (Higuchi et al., 2001; Inoue et al., 2003; Kobayashi et al., 2005; Bashir et al., 2006) e milho (Mizuno et al., 2003; Bashir et al., 2006). Contudo, a secreção de PS ainda não foi caracterizada a nível molecular em nenhuma espécie de gramínea. Uma série de evidências aponta para um mecanismo mediado por vesículas para a secreção de PS (Nishizawa et al., 1987; Sakaguchi et al., 1999; Negishi et al., 2002; Mizuno et al., 2003). De qualquer modo, o PS secretado é capaz de quelar o Fe^{3+} e os complexos Fe(III)-PS então formados são absorvidos por transportadores ortólogos do Yellow Stripe 1 de milho (YS1; Curie et al., 2001).

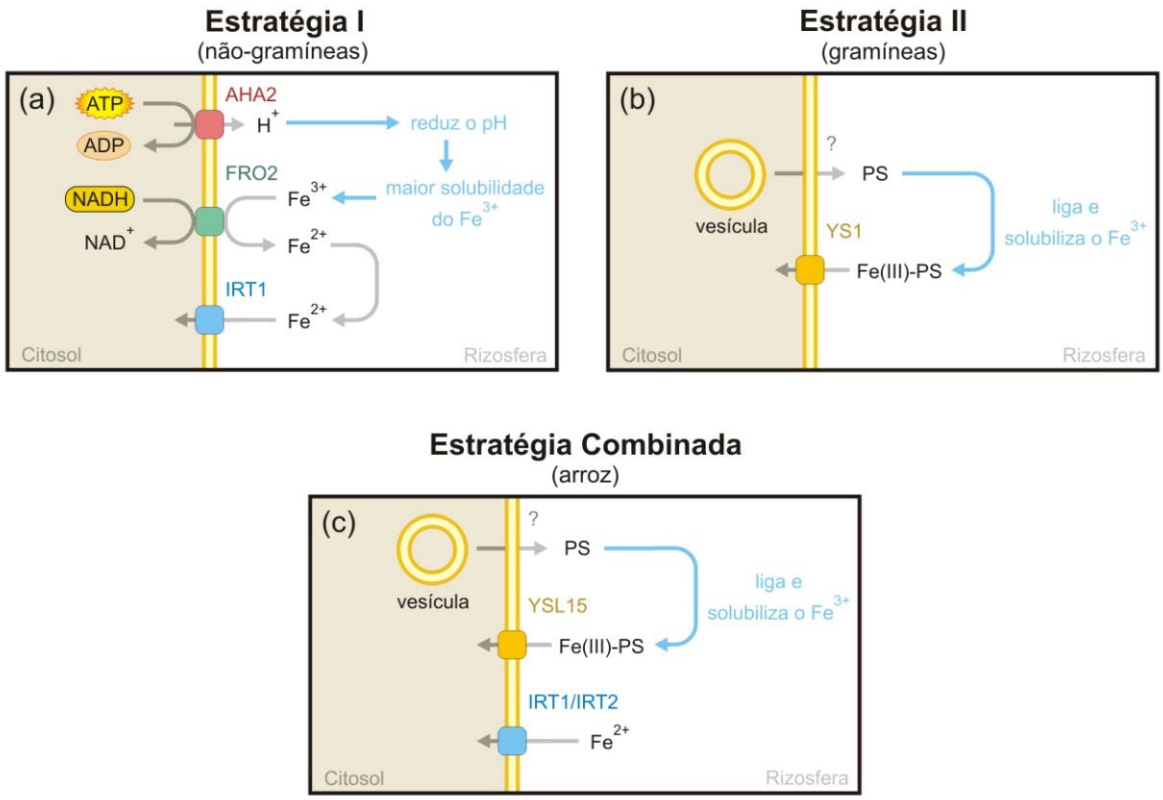


Figura 2. Estratégia para captura de Fe a partir do solo. **(a)** Estratégia I. Próton-ATPases (AHA2) liberam prótons para acidificar a rizosfera e aumentar a solubilidade do Fe^{3+}. O Fe^{3+} solubilizado é reduzido por ferro-redutases ligadas à membrana (FRO2), e o Fe^{2+} é absorvido por transportadores de metal (IRT1). **(b)** Estratégia II. Fitosideróforos (PS) são sintetizados e liberados na rizosfera, possivelmente por um mecanismo mediado por vesículas. Os PS quelam o Fe^{3+} rizosférico, e os complexos Fe(III)-PS são absorvidos por transportadores de oligopeptídeos (YS1). **(c)** Estratégia combinada. Além de capturar Fe^{3+} pela estratégia II, o arroz também pode absorver diretamente o Fe^{2+} por transportadores de metal (IRT1/IRT2). Os nomes dos genes em (a), (b), e (c) se referem respectivamente a *Arabidopsis*, milho e arroz.

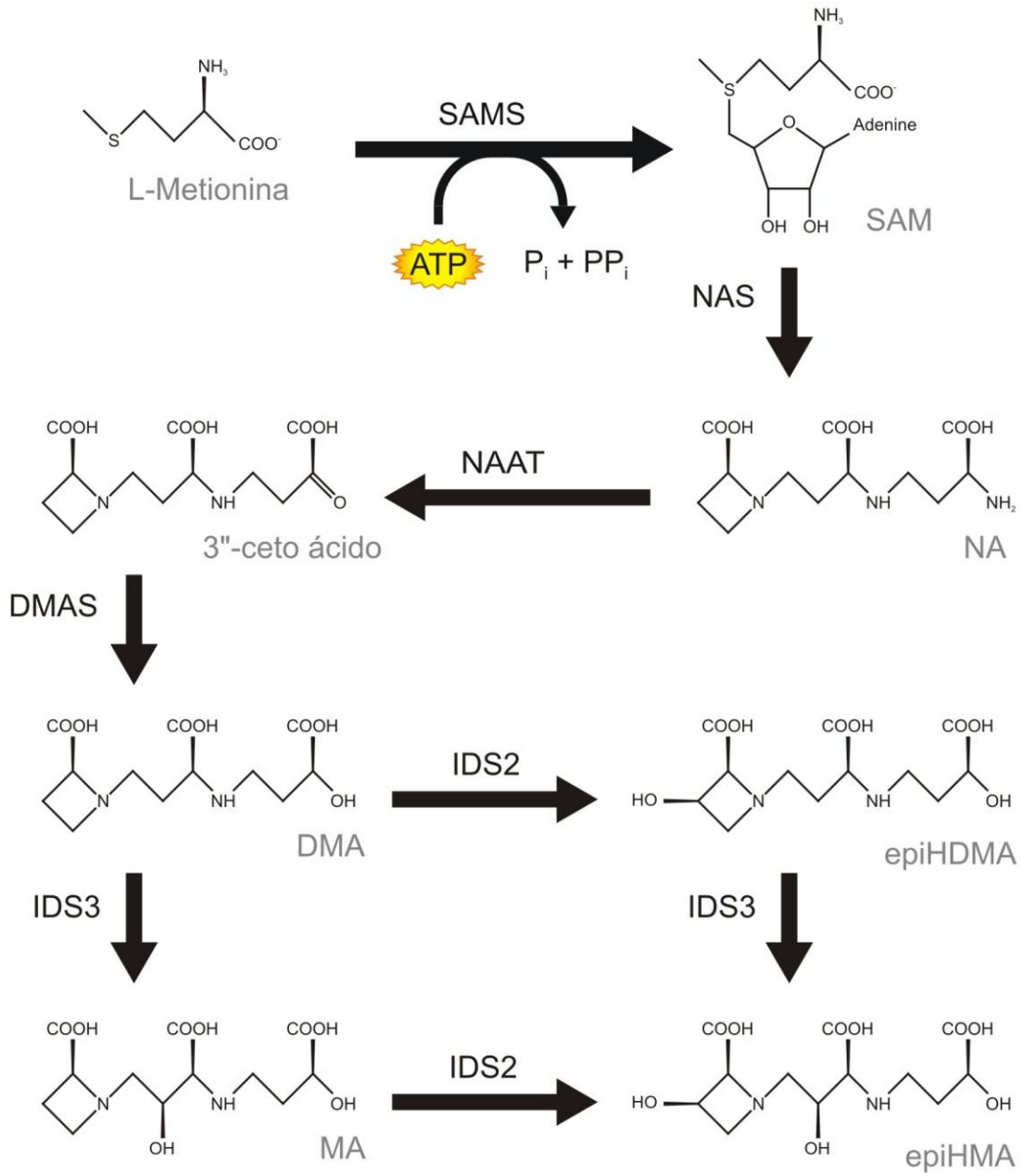


Figura 3. Rota de biossíntese e estrutura molecular dos fitosideróforos da família do ácido mugineico. A L-metionina é adenosilada pela SAMS, que consome ATP e forma a S-adenosil-metionina (SAM). A NAS combina três moléculas de SAM para formar a nicotianamina (NA). A NAAT catalisa a amino transferência da NA e forma o intermediário 3"-ceto ácido, que então é subseqüentemente reduzido pela DMAS para formar o ácido desoximugineico (DMA). As dioxigenases IDS2 e IDS3 catalisam diferentes etapas de hidroxilação para formar os outros fitosideróforos da família do ácido mugineico.

Havia sido aceito que essas duas estratégias eram completamente distintas, considerando-se que tanto o mutante *ys1* de milho (defectivo para absorção de Fe(III)-PS), quanto o mutante *irt1* de *Arabidopsis* (defectivo para absorção de Fe^{2+}) apresentam clorose

grave e morrem ainda como plântulas quando sob deficiência de Fe (Curie et al., 2001; Vert et al., 2002). Contudo, o arroz parece adotar uma **estratégia combinada** (Figura 2; Walker & Connolly, 2008). Apesar do fato de plantas de arroz produzirem e secretarem PS em baixas quantidades quando comparadas a outras gramíneas (Mori et al., 1991), elas são capazes de absorver Fe(III)-PS de modo eficiente através da proteína OsYSL15 (Os02g0650300; Inoue et al., 2009; Lee et al., 2009). Mas diferentemente de outras gramíneas, as plantas de arroz também absorvem Fe^{2+} quando disponível através dos transportadores OsIRT1 (Os03g0667500) e OsIRT2 (Os03g0667300), mesmo que sejam incapazes de induzir a extrusão de prótons e a redução do Fe^{3+} como as não-gramíneas (Ishimaru et al., 2006). Especula-se que essa habilidade de absorver Fe^{2+} evoluiu no arroz como uma adaptação a campos alagados, onde o Fe^{2+} é provavelmente mais abundante que o Fe^{3+} devido às condições anaeróbicas que prevalecem nesses campos (Jeong & Guerinot, 2009).

Captação de zinco pelas plantas

Em condições de suficiência de Zn, acredita-se que a maioria do Zn seja obtida como cátion divalente (Zn^{2+}); e em pH alto, possivelmente também seja obtido como cátion monovalente ($ZnOH^{+}$; Marschner, 1995). Em comparação com as respostas à deficiência de Fe, pouco se sabe sobre os detalhes moleculares da aquisição de Zn a partir do solo em condições de deficiência desse metal. Como dito na seção anterior, a proteína AtIRT1 de *Arabidopsis* é capaz de transportar Zn^{2+}, e mutantes *irt1* têm níveis reduzidos de Zn na raiz quando sob deficiência de Fe (Vert et al., 2002). Contudo, a expressão de AtIRT1 não é induzida por deficiência de Zn e mutantes *irt1* não têm o fenótipo selvagem recuperado por aplicação exógena de Zn em excesso; esses resultados indicam que o Zn é capturado

primariamente por outros transportadores ainda não caracterizados (Walker & Connolly, 2008).

Os PS liberados pelas raízes de gramíneas durante a deficiência de Fe para mobilizar o Fe^{3+} presente no solo também são capazes de quelar outros metais divalentes, incluindo o Zn^{2+} (Benes et al., 1983; Anderegg & Ripperger, 1989). Por esse motivo, foi proposto que as gramíneas também seriam capazes de absorver complexos Zn(II)-PS (Welch, 1995; von Wirén et al., 1996). Em suporte a essa proposta, foi observada a indução de genes que participam da biossíntese de PS e uma maior secreção de PS em raízes de cevada sob deficiência de Zn (Suzuki et al., 2006a). Além disso, foi confirmado que plantas de cevada sob deficiência de Zn absorvem tanto Zn(II)-PS quanto Zn^{2+}, e com taxas de captura maiores detectadas para Zn(II)-PS (Suzuki et al., 2006a). O transportador HvYS1 de cevada é o principal responsável pela captura de Fe(III)-PS, mas análises por eletrofisiologia de oócitos de *Xenopus laevis* indicam que essa proteína não transporta outros íons, o que sugere a ação de outro transportador na absorção de Zn. Em contraste, ZmYS1 de milho foi capaz de absorver em análises semelhantes Fe(III), Fe(II), Ni(II), Zn(II), Cu(II), Mn(II) e Cd(II) quelados por PS (Schaaf et al., 2004; Murata et al., 2006). Consistente com esse resultado, o mutante *ys1* de milho absorve menores quantidades de Zn(II)-DMA do que plantas do tipo selvagem (von Wíren et al., 1996).

Diferentemente de plantas de cevada, a secreção de PS diminui em plantas de arroz sob deficiência de Zn (Suzuki et al., 2005), e essas plantas absorvem menos Zn quando supridas com PS exógeno (Suzuki et al., 2006b), sugerindo uma contribuição relativamente baixa de PS para a captura de Zn em arroz. Além disso, estudos sobre fracionamento dos isótopos em plantas de arroz reportaram um enriquecimento no isótopo leve (^{64}Zn) quando crescidas em hidroponia e um enriquecimento no isótopo pesado (^{66}Zn) quando crescidas em

solo deficiente em Zn (Weiss et al., 2005; Arnold et al., 2010). Esses resultados indicam que as plantas absorvem o Zn na sua forma livre quando em hidroponia, mas absorvem o Zn complexado a algum quelante quando em solo deficiente nesse metal. Logo, ainda não está clara a importância dos PS para a captura do Zn em arroz. De qualquer modo, acredita-se que transportadores pertencentes à família ZIP (e YSL, no caso de gramíneas) possam exercer um papel primário na captura de Zn a partir do solo, porém nenhum membro dessa ou de nenhuma família foi caracterizado como tendo essa função até o momento (Palmgren et al., 2008).

Quelantes de metais pesados

Como foi discutida em seções anteriores, a presença em excesso de íons metálicos na sua forma livre pode ter efeitos nocivos sobre o metabolismo celular; e após serem capturados a partir do solo esses íons metálicos devem ser armazenados e transportados em formas não-reativas. Por isso, espera-se que uma proporção muito pequena do conteúdo intracelular total de metais exista como íons livres: por exemplo, a concentração de Zn^{2+} livre na bactéria *Escherichia coli* foi calculada na ordem de femtomolar (10^{-15} M), apesar de uma concentração intracelular total na ordem de milimolar (10^{-3}; Outten & O'Halloran, 2001). A grande maioria dos íons metálicos que não esteja ocupando sítios em proteínas provavelmente deve estar quelada por ligantes metálicos de baixa massa molecular (Haydon & Cobbett, 2007b). Esses ligantes metálicos podem ter funções intracelulares como quelantes para seqüestrar íons metálicos no citosol ou em compartimentos subcelulares; ou – para metais com baixa solubilidade como o Fe – eles podem ser usados tanto para mobilização do solo quanto para translocação dentro da planta (Haydon & Cobbett, 2007b).

Diversas moléculas de baixa massa molecular já foram descritas como quelantes de metais pesados em plantas, entre elas: a nicotianamina (NA; von Wiren et al., 1999); ácidos orgânicos, como o citrato e o malato (Abadía et al., 2002); as fitoquelatinas (PC; Clemens, 2006); as metalotioneínas (MT; Zhou & Goldsbrough, 1994); o fitato (Rauser, 1999); e a ITP (Iron Transport Protein; Krueger et al., 2002). Além desses, a ferritina (FER) é uma proteína globular de 24 subunidades que forma em seu interior uma cavidade capaz de armazenar até 4.500 átomos de Fe de forma não-tóxica em cloroplastos e mitocôndrias (Briat et al., 2006). Esse grande número de ligantes com diferentes localizações histológicas e subcelulares indica fortemente que a especiação de metal-ligantes nas plantas é dinâmica, mudando entre tecidos, entre organelas e entre estágios do desenvolvimento (Haydon & Cobbett, 2007b).

Assim como ocorre com os transportadores, esses quelantes também apresentam uma amplitude de 'substratos', e a NA é um bom exemplo disso. Esse metabólito é sintetizado por todas as plantas (Figura 3), sendo capaz de quelar (em ordem de menor para maior afinidade): Mn^{2+}, Fe^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} e Fe^{3+} (Curie et al., 2009). A NA não é secretada pelas plantas, e uma série de evidências indicam que esse composto tem como funções principais quelar o Fe nos ambientes simplásticos e durante o transporte de longa distância através do floema (Takahashi et al., 2003; Koike et al., 2005; Curie et al., 2009). A NA também pode ser importante no seqüestro do Fe no vacúolo, pois plantas de tomate (*Solanum lycopersicum* L.) e ervilha (*Pisum sativum* L.) sob excesso de Fe acumulam NA nesse compartimento subcelular (Pich et al., 2001). De modo similar, o fenótipo de mutantes perda-de-função para transportadores FRD (Ferric Reductase Defective) responsáveis por carregar o citrato no xilema indicaram a importância desse ácido orgânico como quelante de Fe nesse tecido (Durrett et al., 2007, Yokosho et al., 2009). Recentemente, a primeira evidência direta e inequívoca da existência do complexo Fe(III)-citrato foi obtida a partir da seiva do xilema de tomate (Rellán-Álvarez et al., 2010).

Um mecanismo proposto para a troca de ligantes do Fe é baseado na maior estabilidade dos complexos Fe(II)-NA no ambiente neutro-para-ácido do floema e na maior estabilidade dos complexos Fe(III)-citrato no ambiente básico do xilema: a simples troca de pH do meio seria suficiente para ocorrer a troca do ligante (Curie et al., 2009). Outro mecanismo envolveria alterações no estado redox do metal quelado: por exemplo, o complexo Fe(II)-NA é muito mais estável do que o complexo Fe(III)-NA (von Wiren et al., 1999; Rellán-Álvarez et al., 2008). Um estudo sobre o fracionamento de isótopos de Fe demonstrou que tecidos jovens de plantas de estratégia I ficam enriquecidos em isótopos leves, o que seria um indício de etapas de redução ao longo da translocação do Fe (esse enriquecimento não foi observado em plantas de estratégia II; Guelke & Von Blackenburg, 2007). Esse resultado e dados de expressão de genes FRO levaram à proposta de que proteínas dessa família tenham um papel nessas etapas de redução relacionadas com troca de ligante e/ou transporte transmembrana (ver seção Família FRO).

Portanto, apesar de todos os avanços recentes na compreensão da homeostase de metais, muitas questões ainda estão para serem respondidas, em particular aquelas relativas ao transporte entre tecidos e a distribuição subcelular. Até agora não está claro como os metais são carregados e descarregados dos tecidos vasculares, o que é uma etapa crítica na translocação dos metais ao longo do corpo da planta (Palmer & Guerinot, 2009). Igualmente, ainda estão para serem totalmente elucidados os sistemas de transporte de metais para a mitocôndria e os cloroplastos, organelas nas quais o Fe e o Zn exercem funções essenciais (Raven et al., 1999; Lister et al., 2002; Moberg et al, 2003). E considerando que esses metais existem em mais de um estado redox, e muitos transportadores e quelantes têm maior afinidade por certas valências, é bem provável que redutases estejam presentes em áreas de transição onde os metais precisem ser reduzidos para transporte e ligação (Jeong & Connolly, 2009). As plantas-modelo têm tido um papel proeminente na elucidação desses processos

relativos à homeostase de metais em plantas, em particular *Arabidopsis thaliana* para as dicotiledôneas e o arroz para as monocotiledôneas.

Arroz

O arroz é uma espécie importante nos estudos sobre a homeostase de metais, pois essa planta possui tanto importância científica, quanto importância econômica. Sua importância econômica provém do fato do arroz ser um dos cereais mais produzidos e consumidos no mundo: foi estimada uma produção mundial de 678 milhões de toneladas em 2009 (http://www.fao.org), e metade da população mundial tem no arroz sua principal fonte de alimentação. Além disso, o Brasil é o maior produtor não-asiático de arroz, e o Rio Grande do Sul é responsável por metade da produção nacional (http://www.irga.rs.gov.br). Já a sua importância científica se deve ao fato de ter o menor genoma haplóide dentre as gramíneas, com um tamanho estimado de 430 Mb distribuídas entre 12 cromossomos (2n = 24, Burr, 2002). Por essa razão, o primeiro genoma de monocotiledôneas a ser seqüenciado foi o do arroz, e diversos recursos estão disponíveis atualmente para estudos de genética clássica e reversa, tornando essa uma espécie-modelo para o estudo de gramíneas (Krishnan et al., 2009).

Um desses recursos é o banco de mutantes perda-de-função pela inserção do retrotransposon endógeno *Tos17* disponibilizado pelo Rice Genome Resource Center (http://www.rgrc.dna.affrc.go.jp) do National Institute of Agrobiological Sciences do Japão. A atividade do *Tos17* é estritamente regulada: sua transposição é ativada por cultura de tecido e inativada pela regeneração (Hirochika, 1997). Além disso, esse retrotransposon tem preferência por regiões de baixo número de cópias do genoma, que em geral são ricas em

genes (Yamazaki et al., 2001). Essas características tornam esse elemento extremamente útil para estudos de genética funcional de arroz.

Além disso, o arroz também tem sido um dos alvos preferenciais em empreendimentos internacionais de biofortificação (i.e., o desenvolvimento de linhagens com maior conteúdo de nutrientes biodisponíveis nas suas partes comestíveis), pois os seres humanos consomem principalmente o seu endosperma amiláceo, o qual é rico em calorias, mas pobre em nutrientes, em especial vitamina A, Fe e Zn. A deficiência de micronutrientes é um dos mais sérios desafios globais para a humanidade, e esses três nutrientes constituem as principais deficiências nutricionais humanas no mundo. Desse modo, a produção de linhagens de arroz com endosperma enriquecido em Fe e Zn pode ter um impacto positivo sobre a saúde de populações humanas (White & Broadley, 2009).

Genes relacionados à homeostase de metais em arroz

Com o fim do seqüenciamento do genoma do arroz, tanto da subespécie *indica* (Yu et al. 2002) como da subespécie *japonica* (Goff et al. 2002), foi possível realizar buscas de genes relacionados com a homeostase de Fe e Zn no genoma do arroz. Um levantamento inicial realizado pelo nosso grupo permitiu a identificação de 43 genes potencialmente envolvidos na homeostase do Fe (incluindo genes envolvidos nas estratégias I e II de captura de Fe): 18 genes YSL, 2 FRO, 13 ZIP, 8 NRAMP e 2 FER (Gross et al. 2003). Um levantamento mais recente utilizando dados da literatura em conjunto com buscas nos bancos de dados disponíveis permitiu a identificação de um número ainda maior de genes (Tabela 2; dados não-publicados). Muitos desses genes putativos ainda não foram funcionalmente caracterizados, incluindo a maioria dos genes pertencentes à família ZIP e os dois genes da família FRO encontrados no genoma de arroz.

Tabela 2. Genes encontrados no genoma de arroz relacionados ou possivelmente relacionados com a homeostase de Fe e Zn*.

Nome do gene	Nº de membros	Proteína codificada	Referência
Biossíntese de NA e PS			
OsNAS	3	Nicotianamine synthase	Inoue et al. (2003)
OsNAAT	6	Nicotianamine aminotransferase	Inoue et al. (2008)
OsDMAS	1	Deoxymugineic acid synthase	Bashir et al. (2006)
Regulação gênica relativa à deficiência de Fe			
OsIRO	1	bHLH transcription factor	Ogo et al. (2006)
OsIDEF	1	Fe deficiency-responsive element factor	Kobayashi et al. (2007)
Captura e translocação de metal			
OsZIP	14	Zinc and Iron-regulated transporter	Ramesh et al. (2003)
OsIRT	2	Iron-regulated transporter	Bughio et al. (2002)
OsZIFL	13	Zinc-induced facilitator	Haydon & Cobbett (2007a)
OsNRAMP	8	Natural resistance associated macrophage protein	Zhou & Yang (2004)
OsMTP	1	Metal tolerance protein	van der Zaal et al. (1999)
OsHMA	9	Heavy metal-associated domain	Mills et al. (2003)
OsFPN / OsMARL	1 / 2	Ferroportin / Multiple antibiotic resistance like	Schaaf et al. (2006)
Redução do Fe^{+3}			
OsFRO	2	Ferric reductase oxidase	Ishimaru et al. (2006)

Tabela 2. Continuação.

Nome do gene	Nº de membros	Proteína codificada	
Captura e translocação de metal-PS			
OsYSL	18	Yellow stripe-like metal-PS transporter	Koike et al. (2004)
Armazenamento de Fe^{+2}			
OsFER	2	Ferritin	Stein et al. (2009)
Efluxo ou influxo de metal para organelas			
OsMIR	1	Mitochondrial iron-regulated gene	Ishimaru et al. (2009)
OsPIC	2	Permease in chloroplasts	Duy et al. (2007)
OsVIT	2	Vacuolar iron uptake transporter	Kim et al. (2006)

*Levantamento realizado com base em dados da literatura e em buscas *in silico* de bancos de dados utilizando genes de outras espécies como seqüências *query*.

Família ZIP

A família ZIP (ZRT, IRT-like protein) foi nomeada com base nos seus três membros fundadores: ScZRT1 e ScZRT2, respectivamente os transportadores de alta e baixa afinidade de Zn em levedura (*Saccharomyces cerevisiae*; Zhao & Eide, 1996a; 1996b), e AtIRT1, um transportador de Fe em *Arabidopsis* (Eide et al., 1996). Essa família está presente em todos grandes grupos filogenéticos: bactérias, arqueas, fungos, plantas e animais (Guerinot, 2000). Baseado nas 15 proteínas ZIP seqüenciadas até aquele momento, Eng et al. (1998) descreveu as características típicas dessas proteínas como tendo 8 domínios transmembrana e extremidades amino- e carboxi-terminais voltadas para o meio extracelular. Essas proteínas variavam de 233 a 477 aminoácidos, e grande parte dessa variação podia ser atribuída a chamada 'região variável' entre os domínios transmembrana III e IV. Além disso, vários membros dessa família continham domínios ricos em histidina nessa região variável.

Uma análise filogenética subseqüente por Guerinot (2000) incluiu as 29 proteínas ZIP depositadas no GenBank até aquele ano, o que permitiu a distinção de dois grupos: a subfamília I (principalmente proteínas de plantas e fungos, e algumas de animais) e a subfamília II (somente proteínas de animais). A análise mais recente a incluir todas as proteínas ZIP no banco não-redundante de seqüências protéicas do GenBank incluiu 86 proteínas ZIP e resultou na adição de duas novas subfamílias (Gaither & Eide, 2001). A subfamília LZT (LIV-1 subfamily of ZIP zinc Transporters; Taylor & Nicholson, 2003) foi nomeada pela proteína LIV-1 (Slc39a6 ou HsZIP6) que é expressa em níveis elevados nos tecidos de certos cânceres de mama metastáticos (Manning et al., 1988): essa subfamília continha duas proteínas de *Arabidopsis*. Já a subfamília gufA foi designada com base na proteína gufA (gene of unknown function A) de *Myxococcus xanthus* (McGowan et al. 1993) e abrangia proteínas de procariotos e eucariotos, mas nenhuma de plantas.

Família ZIP em arroz

Já existem informações publicadas sobre alguns genes da família ZIP em arroz, tais como *OsIRT1* e *OsIRT2*: esses genes são induzidos em raízes sob deficiência de Fe, e as proteínas codificadas por eles se localizam na membrana plasmática e transportam Fe e Cd, mas não Cu ou Mn (Bughio et al., 2002; Ishimaru et al., 2006; Nakanishi et al., 2006). A expressão heteróloga de OsIRT1 em leveduras defectivas para a aquisição de Zn não recuperou o crescimento dessas leveduras (Ishimaru et al., 2006), porém plantas super-expressando *OsIRT1* apresentaram maior sensibilidade à presença de Zn no meio (Lee & An, 2009). Logo, ainda não está claro se OsIRT1 é capaz de transportar Zn ou não.

Lee & An (2009) observaram que *OsIRT1* é induzido em parte aérea sob deficiência de Fe, e também em raiz sob deficiência de Fe, Mn, Zn e Cu. Este mesmo trabalho detectou a

expressão de *OsIRT1* no estelo e na epiderme da raiz, além de em células companheiras no colmo. Portanto, essa proteína parece ter alguma função também no transporte interno de metais.

Ramesh et al. (2003) demonstraram que *OsZIP1* (Os01g0972200) é expresso em parte aérea e raiz, e é induzido sob deficiência de Zn em raiz. Essa proteína parece não transportar somente Zn, como também Mg e Cd. Em contraste, baixos níveis de transcritos de *OsZIP2* (Os03g0411800) foram detectados em raiz sob deficiência de Zn, e essa proteína não apresentou nenhuma capacidade de transporte sob as condições testadas (Ramesh et al., 2003).

O gene *OsZIP3* (Os04g0613000) também foi caracterizado por Ramesh et al. (2003), e a proteína codificada por esse gene parece ser bastante específica para o transporte de Zn. Contudo, os dados de expressão (dados de *Northern blot*) apresentados nesse estudo estão em contradição com aqueles apresentados posteriormente por Ishimaru et al. (2005), que não detectaram transcritos desse gene nem em parte aérea, nem em raiz (dados de qPCR).

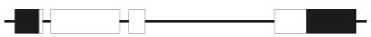
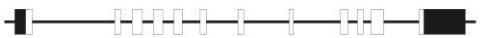
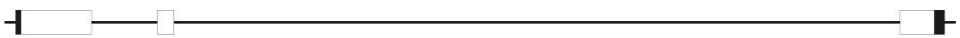
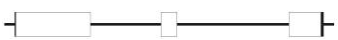
Ishimaru et al. (2005) identificou num banco de dados de cDNA quatro outros genes ZIP em arroz: *OsZIP4* (Os08g0207500), *OsZIP5* (Os05g0472700), *OsZIP6* (Os05g0164800) e *OsZIP7* (Os05g0198400). Dados de microarranjo indicaram que *OsZIP4* era altamente induzido em raiz sob deficiência de Zn e que *OsZIP4*, *OsZIP5* e *OsZIP7* eram induzidos em parte aérea sob deficiência de Zn. A proteína OsZIP4 se localizou na membrana plasmática e, ao ser expressa em leveduras, foi capaz de transportar Fe e Zn, mas não Mn ou Cu. Contudo, o padrão espacial de expressão desse gene sugere um papel no transporte interno desses metais: folhas (feixes vasculares e células do mesófilo), colmo (floema), meristema apical e raiz (feixes vasculares, especialmente o floema). A super-expressão de *OsZIP4* causou

inibição do crescimento em plantas transgênicas, provavelmente devido ao acúmulo de Zn nas raízes e aos baixos níveis de Zn na parte aérea (Ishimaru et al., 2007).

Finalmente, Yang et al. (2009) clonaram os genes *OsZIP7a* (um variante de *OsZIP7* da cultivar Jiucaiqing) e *OsZIP8* (Os02g0196000), e o primeiro complementou leveduras defectivas para absorção de Fe, enquanto que o segundo o fez para leveduras defectivas em absorção de Zn. Em conformidade, *OsZIP8* foi induzido em raiz sob deficiência de Zn e *OsZIP7a* foi induzido em raiz sob deficiência de Fe.

Uma busca extensiva nos bancos de dados disponíveis para seqüências genômicas, de cDNAs e *contigs* de ESTs de arroz realizada pelo nosso grupo resultou na identificação de 16 genes da família ZIP em arroz, incluindo *OsIRT1* e *OsIRT2* (Tabela 3; dados não-publicados). Isso porque um par de genes identificado por Gross et al. (2003) na realidade correspondia a duas seqüências parciais de um mesmo gene: *OsZIP9* e *OsZIP10* são o mesmo gene. E o mesmo ocorreu para o par de genes *OsZIP14* e *OsZIP15*, identificados como dois genes distintos por Chen et al. (2008). De qualquer modo, esses resultados deixam claro que a caracterização funcional da família ZIP de arroz ainda está longe de estar completa.

Tabela 3. Genes da família ZIP no genoma de arroz.

Gene	TIGR Locus	RAP2 Locus	cDNA	TMD*	Tamanho (aa)	Representação esquemática†
OsIRT1	Os03g46470.1	Os03g0667500	AK107681	8	374	
OsIRT2	Os03g46454.1	Os03g0667300	PUT-163a-OsJG-64750‡	-	396	
OsZIP1	Os01g74110.1	Os01g0972200	AY302058	9	352	
OsZIP2	Os03g29850.1	Os03g0411800	AK121551	9	358	
OsZIP3	Os04g52310.1	Os04g0613000	AK069804	7	364	
OsZIP4	Os08g10630.1	Os08g0207500	AK105258	8	396	
OsZIP5	Os05g39560.1	Os05g0472700	AK070864	8	353	
OsZIP6	Os05g07210.1	Os05g0164800	AK103730	7	395	
OsZIP7	Os05g10940.1	Os05g0198400	AK071272	6	384	
OsZIP8	Os02g10230.1	Os02g0196000	AK068640	8	276	
OsZIP9	Os05g39540.1	Os05g0472400	AK243641	8	362	
OsZIP11	Os05g25194.2	Os05g0316100	AK066887	13	577	
OsZIP12	Os06g37010.2	Os06g0566300	AK241111	7	410	
OsZIP13	Os07g12890.1	Os07g0232800	AY324148	8	390	
OsZIP14	Os08g36420.2	Os08g0467400	AK070501	6	498	
OsZIP16	Os08g01030.1	Os08g0100200	Nenhum	8	282	

*TMD é o número predito de domínios transmembrana (valor de corte = 0.5). †Íntrons são representados por linhas, regiões não-traduzidas por caixas pretas e regiões codificadoras por caixas brancas. Como não foi encontrado nenhum cDNA ou contig de EST para OsZIP16, só está representada em cinza a região codificadora predita. ‡Código relativo a um *contig* de ESTs.

Família FRO

Duas abordagens distintas permitiram a identificação do primeiro membro da família FRO em plantas: (1) a identificação do gene mutante em plantas *frd1* (ferric reductase defective 1) que não possuíam atividade de redutase férrica induzida por deficiência de Fe nas suas raízes (Yi & Guerinot, 1996), e (2) amplificação por PCR baseada na similaridade com as redutases férricas de leveduras (FRE) e com a oxidase humana $Gp91^{phox}$ (Robinson et al., 1997). O gene identificado foi o *AtFRO2* de *Arabidopsis*, que é o principal responsável pela atividade de redutase observada na raiz dessa espécie de estratégia I para aquisição de Fe (Figura 2; Robinson et al., 1999). O seqüenciamento do genoma de *Arabidopsis* revelou a presença de oito genes FRO no total (Jeong & Connolly, 2009), e genes codificando proteínas FRO de outras espécies de estratégia I também já foram clonados: *PsFRO1* de ervilha (Waters et al., 2002), *SlFRO1* de tomate (Li et al., 2004), *CsFRO1* de pepino (*Cucumis sativus* L.; Waters et al., 2007) e *AhFRO1* de amendoim (*Arachis hpyogaea* L.; Ding et al., 2009).

As proteínas FRO pertencem à superfamília dos flavocitocromos, e acredita-se que a função delas seja transferir elétrons através de membranas para reduzir quelatos de Fe (talvez de Cu também) utilizando dois grupos heme intramembrana (Robinson et al., 1999). Esses grupos heme são ligados a dois grupos bis-heme: pares de resíduos de histidina altamente conservados (Figura 4A; Finegold et al., 1996). Outras seqüências conservadas nas proteínas FRO são: o motivo de ligação a FAD (HPFT), o motivo de ligação a NADPH (GPYG) e o motivo de oxirredutase, todos presentes no grande *loop* citosólico entre os domínios

transmembrana IV e V da proteína AtFRO2 que possui oito domínios transmembrana no total (Figura 4B; Schagerlof et al., 2006).

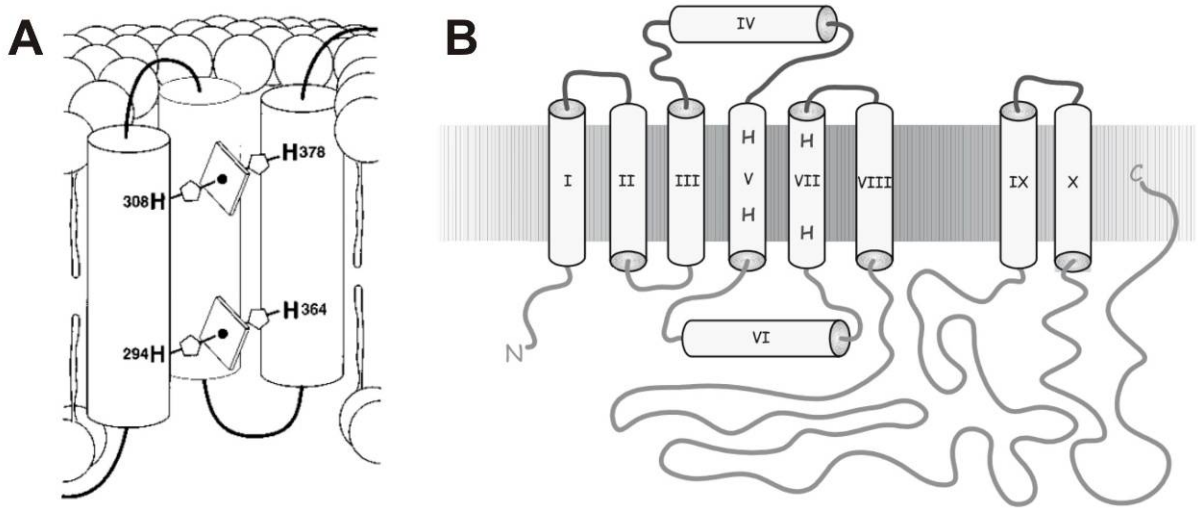


Figura 4. Características estruturais das proteínas FRO. **A.** Modelo estrutural das histidinas intramembrana coordenando os dois grupos heme. A membrana plasmática é representada por duas camadas de círculos com caudas para indicar a bicamada fosfolipídica. Os cilindros representam três α-hélices transmembrana consecutivas. Os grupos imidazol das histidinas coordenando o grupo heme estão numerados de acordo com sua localização na proteína FRE1 de levedura. Os grupos heme são representados por quadrados contendo um ferro hexacoordenado (pontos negros; Finegold et al., 1996) **B.** Modelo da topologia de AtFRO2 baseado em dados experimentais (Schagerlof et al., 2006). A posição da porção C-terminal não pôde ser determinada experimentalmente. Os cilindros representam α-hélices transmembrana preditas *in silico*. As quatro letras H indicam a posição das histidinas ligantes dos grupos heme.

Diversos trabalhos têm demonstrado que as proteínas FRO devem ter um papel também na distribuição de Fe e Cu dentro da planta. *PsFRO1* é expresso em toda a raiz e também em folhas (Waters et al., 2002), e *SlFRO1* é expresso em raiz e parte aérea (Li et al., 2004). O gene *AtFRO3* é induzido por deficiência de Fe e é expresso no cilindro vascular de raiz e parte aérea, e fusões AtFRO3-GFP se co-localizam com marcadores mitocondriais (Mukherjee et al., 2006; Dinneny et al., 2008; Jeong & Connolly, 2009). E pelo menos outros

três genes FRO de *Arabidopsis* – *AtFRO6*, *AtFRO7* e *AtFRO8* – são expressos em tecidos verdes (Mukherjee et al., 2006). A proteína AtFRO6 se localiza na membrana plasmática, e protoplastos de mutantes *fro6* têm menor atividade de redutase férrica (Feng et al., 2006; Jeong & Connolly, 2009). Já a proteína AtFRO7 se localiza no cloroplasto, e cloroplastos isolados de mutantes *fro7* também apresentam menor atividade de redutase férrica e menor conteúdo de Fe (Jeong et al., 2008). Por fim, a proteína AtFRO8 se localiza na mitocôndria, e esse gene é altamente expresso em folhas senescentes (Zimmerman et al., 2004; Jeong & Connolly, 2009). Esses resultados, em conjunto, indicam que esses três genes têm funções distintas no transporte de Fe em tecidos verdes de *Arabidopsis*.

Família FRO em arroz

Uma busca recente nos bancos de dados disponíveis levou à identificação de membros da família FRO em pelo menos 21 espécies de planta, incluindo espécies de estratégia II como o arroz, o milho e o sorgo (*Sorghum bicolor* L.). Essa parece ser uma família gênica extremamente bem conservada, pois – à exceção de uma – todas as proteínas encontradas apresentaram os motivos típicos de membros da família FRO conservados (ver capítulo 2).

O genoma do arroz possui dois genes FRO: *OsFRO1* (Os04g0444800) e *OsFRO2* (Os04g0578600), identificados por Gross et al. (2003). O único trabalho até o momento com dados experimentais relativos a esses dois genes é o de Ishimaru et al. (2006). Este artigo demonstrou que plantas de arroz não induzem atividade de redutase na raiz durante a deficiência de Fe, o que está de acordo com a expressão dos genes FRO somente em parte aérea. Transcritos de *OsFRO1* foram detectados em folhas de plantas sob deficiência de Zn, Mn e Cu; enquanto que transcritos de *OsFRO2* foram detectados em folhas sob deficiência de

Fe (Ishimaru et al., 2006). De qualquer maneira, ainda não está totalmente claro qual o papel dessas redutases em arroz.

Objetivos

O objetivo deste trabalho é a caracterização funcional de genes das famílias ZIP e FRO em plantas de arroz. Para atingir este objetivo, lançamos mão das seguintes abordagens:

• Levantamento dos genes dessas famílias e das seqüências genômica, de cDNA, codificadora e de proteína em diferentes bancos de dados disponíveis.

• Busca de mutantes perda-de-função dos genes dessas famílias para análises de genética reversa.

• Análise da expressão gênica dos membros dessas famílias em diferentes órgãos e tratamentos.

Capítulo 1:

OsZIP3 is involved in viability and Zn concentration of rice pollen

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Abstract

Zinc (Zn) is one of the essential chemical elements required by plant metabolism, however little is known about the molecular players involved in Zn homeostasis. The ZIP family members have been characterized as Zn and/or Fe transporters in different species. Three *Tos17* insertion loss-of-function rice mutants were obtained for three different ZIP family genes and homozygous plants for the insertions were isolated. The insertion of *Tos17* clearly reduced the transcription level of the disrupted genes. None of the mutants' seedlings differed visually from wild type seedlings under Zn deprivation or in their ability to accumulate Zn in leaves under different supplied Zn concentrations. Mature plants were evaluated for their agronomic traits and their seed metal concentrations determined. The *zip4* and *zip7* mutants did not present drastic phenotypic alterations, in contrast to the *zip3* mutant, which presented severely reduced seed yield per plant due to a larger number of empty spikelets. Further investigation indicated that pollen viability and pollen grain Zn concentration was reduced in *zip3* plants. Transcripts of *OsZIP3* were mainly detected during the R3 stage (panicle exertion), which is the stage when pollen formation occurs. Our results suggest that OsZIP3 has a role in pollen grain Zn loading.

Introduction

Zinc (Zn) is one of the essential chemical elements required by the metabolism of living organisms, mainly because of its role as a cofactor and structural stabilizer in many proteins (Coleman, 1998). In humans, the Zn status affects multiple physiologic and metabolic functions, such as physical growth, immuno-competence, reproductive function, and neuro-behavioral development (IZiNCG, 2004). When the supply of dietary Zn is insufficient to support these functions, biochemical abnormalities and clinical signs may develop. Zinc deficiency is one of the most widespread nutritional deficiencies around the world (Prasad, 2003). Therefore, there is considerable interest in developing crops with enhanced Zn content in their edible parts (Palmgreen et al., 2008).

Along with its importance in human nutrition, Zn is also a micronutrient required for plants to successfully complete their life cycles (Marschner, 1995; Broadley et al., 2008). Zinc is crucially involved in key metabolic processes, such as photosynthesis and gene expression (Barak & Helmke, 1993). When Zn supply is inadequate, one or more physiological functions are compromised and plant development is impaired (Alloway, 2004). On the other side, free Zn can be detrimental when present in high concentrations, probably due to the displacement of other metal ions from protein binding sites (Chaney, 1993). Thus, Zn uptake and distribution throughout the plant must be strictly regulated to ensure sufficient levels and avoid potentially toxic overaccumulation (Palmgreen et al., 2008).

Zinc, like other metal ions, is unable to cross biological membranes through passive diffusion (Guerinot & Eide, 1999). In order to enter the plant, Zn has to be actively absorbed as free Zn^{+2} or as Zn-phytosiderophore complexes from the soil solution across the root epidermal cells membranes (Suzuki et al., 2008). Subsequently, every time Zn leaves or enters apoplastic compartments, such as in xylem loading and unloading, there must be an

active membrane transport mechanism involved (Palmgreen et al., 2008). The same applies when symplastic Zn is transported across organellar membranes. These distinct processes suggest that there may be specific Zn transporters with different roles in its distribution process (Guerinot & Eide, 1999).

In fact, a number of transmembrane transporters involved in metal ion homeostasis were identified in the last decades and among them are the genes belonging to the ZIP family (Guerinot, 2000). The acronym ZIP stands for 'ZRT, IRT-like Protein' because these were the first members of this family to be characterized: *ZRT1* and *ZRT2* (Zinc-Regulated Transporter) in yeast (*Saccharomyces cerevisiae*; Zhao et al., 1996a; Zhao et al., 1996b) and *IRT1* (Iron-Regulated Transporter) in *Arabidopsis thaliana* (Eide et al., 1996). The ZIP family has representatives not only in several plant species, but also in archaea, fungi, bacteria and animals (Gaither & Eide, 2001). It was predicted that all ZIP proteins are integral membrane proteins, and most of them were predicted to have eight transmembrane domains and extracytoplasmic terminal ends (Guerinot, 2000). The overall length variability found in this family is largely due to a variable potential histidine-rich metal-binding domain between transmembrane domains III and IV (Guerinot, 2000).

At least sixteen ZIP family genes have been identified in the rice genome (Gross et al., 2003; Chen et al., 2008). The *OsIRT1* gene is the most similar to the ferrous iron (Fe^{+2}) transporter *AtIRT1* from *Arabidopsis thaliana* and was the first rice ZIP gene to be cloned (Bughio et al., 2002). As predicted, *OsIRT1* and its paralogue *OsIRT2* were shown to be Fe^{+2} transporters (Ishimaru et al., 2006). The *OsZIP1*, *OsZIP2* and *OsZIP3* genes were identified through homology with Zn transporters from yeast and other plant species (Ramesh et al., 2003). Other four genes of the ZIP family exhibited similarity to *OsIRT1* and were later

isolated: *OsZIP4*, *OsZIP5*, *OsZIP6* and *OsZIP7* (Ishimaru et al., 2005). *OsZIP8* and the variant *OsZIP7a* (from Jiucaiqing cultivar) were cloned by Yang et al. (2009).

When expressed in a Zn-uptake-deficient yeast strain *OsZIP1*, *OsZIP3, OsZIP4* and *OsZIP8* could reverse the growth defect in Zn-limited media, while *OsZIP4* and *OsZIP7a* could do the same for an Fe-uptake-deficient yeast strain in Fe-limited media. OsZIP1 could also transport magnesium (Mg) and cadmium (Cd). These results suggest that these genes are indeed functional transporters of these respective metal ions (Ramesh et al., 2003; Ishimaru et al., 2005; Yang et al., 2009). The transcription of different ZIP genes in plant species appears to be induced by Zn and/or Fe deficiency, although several plant members of the family were shown to be able to transport copper (Cu), manganese (Mn) and the non-essential ion Cd (Grotz et al., 1998; Korshunova et al., 1999; Pedas et al., 2008).

In this study, the expression of six ZIP genes was profiled in seedlings' roots and shoots, as well as in reproductive organs. Three *Tos17* insertional knockout mutants for different ZIP genes were identified and characterized for their metal content, agronomic traits and gene expression. The *zip3* mutant presented low fertility in field conditions and was further evaluated: the pollen viability and pollen grain Zn concentration were decreased in this mutant. The *OsZIP3* gene was most expressed in panicles during the reproductive stage when pollen formation occurs, and these results suggest a role for OsZIP3 in transporting Zn into pollen grains.

Material & Methods

1. *Tos17* mutant lines identification and homozygous isolation

Search for rice *Tos17* insertion mutant lines was performed in the Rice Genome Resource Center bank website (www.rgrc.dna.affrc.go.jp), and mutants found for three different ZIP genes were requested. Obtained seeds were germinated in the laboratory and genomic DNA of seedlings from segregant lines was extracted (Dellaporta et al., 1983). Plants were screened for *Tos17* insertion homozygosis using a PCR-based strategy, with two complementary primers annealing in the genomic region of the gene and another primer annealing in the 3´-*Tos17* tail (primers are listed in Table 1 and indicated in Fig. 1A). Identified homozygous plants were grown in a greenhouse to provide seeds for the following experiments.

2. Treatments and plant material.

Seeds were surface sterilized and germinated in filter paper soaked with water in Petri dishes for one week (at 28ºC, 16 h of light, 40 μmol m^{-2} s^{-1} light intensity) and seedlings were transferred to plastic pots containing 600 ml of a nutrient solution (Yoshida et al., 1972) for 15 days. After that period, plants were subjected to the same nutrient solution with the respective treatments: control (2 μM of $ZnSO_4$) and Zn deprivation (without $ZnSO_4$) for 7 days. The growth medium in all pots was changed every 5 days. Leaves and roots were separately collected and stored at –80°C until further analyses.

Table 1. List of primers used in this study.

Name	Forward (5' → 3')	Reverse (5' → 3')	Amplicon size (bp)
Homozygous identification			
zip3	ACCAGAAGATGGAGGCAATG	GGTGGCAATAGAAGGCAAAA	578
zip4	ACATGTCCCTGGTCGATCTC	ATTTGCAGCTCTTGGTTGCT	166
zip7	GAGGACGACAACGACAACAA	AATCCAAATGTCACGCCTTC	660
Tos17 tail	AGGTTGCAAGTTAGTTAAGA	-	
Quantitative PCR			
OsZIP1	CTCTTCAAGTTCCTCGCCGTCCT	GCGCCACGATTAATGAATGGGGTG	211
OsZIP2	GCTCATGATTTCGGACGTTT	CCCAAAATCCATGGAAACAA	177
OsZIP3	GCATAGATCTTCAGAATAACAGAGGA	TCACCTGAGATAAGCTTTGGTTT	156
OsZIP4	GATTCTTGGGCAAATGGTGT	ACAACGCTGGGGATTATTTG	258
OsZIP5	CAGGAATGGCAGGTTTTTGT	AGTTTCAACCAACGGAGTGG	113
OsZIP6	GCAACCAGAGTGAAATACGG	AGGAGACGAAAATGGCTCA	154
OsZIP7	TGCACAACAACGGATACAGA	TCAGCCAACAACACTCTCCA	251
OsUBQ	AACCAGCTGAGGCCCAAGA	ACGATTGATTTAACCAGTCCATGA	77
OsACT	TTCTTCGGACCCAAGAATGC	AACAAAAGAGAGAACAAGCAGGAG	72

For analysis of metal concentrations in seedlings, wild type and mutant seedlings were grown in plastic pots containing 600 ml of a nutrient solution (Yoshida et al., 1972) for 15 days (at 25±2°C, 16 h of light, 160 μmol m^{-2} s^{-1} of light intensity). After this period, plants were subjected to the same nutrient solution with the respective treatments: 0.02 μM, 0.2 μM and 2 μM of $ZnSO_4$ for 40 days. The nutrient solution in all pots was changed every 5 days. Leaves and roots were collected and had their metal concentrations determined by the particle-induced x-ray emission (PIXE) technique.

For analysis of gene expression during reproductive stages, wild type and mutant plants were cultivated in soil-filled tanks under field conditions. The distinct reproductive stages were recognized according to Counce et al. (2000). Flag leaves and panicles were harvested at R3 (panicle exertion), R4 (anthesis), R5 (grain filling) and R7 (grain maturity) stages. Panicles at the R4 stage were dissected into palea, lemma, anther and carpel, immediately frozen and stored at –80ºC.

3. Agronomic traits

Plants were cultivated in soil-filled tanks under field conditions and had their agronomic traits evaluated at maturity. Harvest index was calculated as the ratio of total seed yield to total aboveground biomass. Six panicles from each plant were used to estimate the following parameters: the weight of all filled spikelets produced per panicle, and the proportion of filled and empty spikelets in each panicle. In order to estimate total seed metal content per panicle, seed metal concentrations were determined by the inductively coupled plasma optical emission spectroscopy (ICP-OES) technique, and values obtained were multiplied by the total seed dry weight per panicle.

4. Elemental analysis by PIXE and ICP-OES

Leaves collected for PIXE analysis were dried at 60ºC, ground in liquid N_2 and pressed into 1 cm-diameter pellets. Analyses by PIXE were carried out at the 3 MV Tandetron accelerator facility at the Physics Institute at the Universidade Federal do Rio Grande do Sul, Brazil and all measurements were performed according to Silveira et al. (2007). Seed elemental analyses using ICP-OES were performed as described in Sperotto et al. (2009).

5. Pollen viability and Zn concentration

Panicles at R4 stage (anthesis) were collected from wild type and *zip3* plants. Their anthers were harvested and immediately used in the pollen viability assay. Anthers from one panicle were squashed on a slide glass in 1% I_2-KI solution. After the debris was removed by decantation, 300 pollen grains were evaluated for pollen viability under a Sudar light microscope at 10×100 magnification (Polskie Zaklady Optyczne, Warsaw, Poland). Pollen grains fully stained were scored as normal. Aborted, small (half size of a normal grain or smaller) and unstained grains were scored as sterile (Fig. 7A; Yamagata et al., 2007).

To determine Zn concentration in reproductive organs and pollen grains, panicles at the R4 stage from field-grown plants were collected and dissected in the different organs. The anthers were lyophilized and gently crushed to separate the pollen samples from the anthers. Samples (5-10 mg) were microdigested using HNO_3 and H_2O_2, and the precipitate dissolved in HNO_3 1%. The Zn concentration was assessed by the Zincon method (Choi et al, 2007), and calculated using a standard curve prepared with known Zn concentrations. All samples were read at 620 nm using a Smartspec Plus Spectrophotometer (Bio Rad, Hercules, USA).

6. RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated using Trizol® (seedlings' leaves and roots) or Concert™ (seeds, flag leaves, panicles and reproductive organs), following manufactor's instructions (Invitrogen). RNA integrity was checked in 1.0% agarose gel, RNA purity was estimated from the A_{260}/A_{280} absorbance ratio and quantification performed using Qubit™ (Invitrogen).

One microgram of total RNA was treated with RNAse-free DNAse I (Invitrogen) and reverse transcribed using M-MLV (Invitrogen) by priming with $Oligo(dT)_{30}$.

Quantitative polymerase chain reactions (qPCR) were performed in an ABI 7500 (Applied Biosystems), using SYBR® Green (Invitrogen) to monitor double strand DNA synthesis. The following standard thermal profile were used for all qPCRs: 95°C for 5 min; 40 cycles of 95°C for 15 sec, 60°C for 10 sec, 72°C for 15 sec, and a final extension step at 60°C for 35 sec. PCR efficiency values (E) were obtained from the exponential phase of each individual amplification plot using the equation $(1 + E) = 10^{slope}$ (Ramakers et al., 2003) and used to normalize each reaction. In order to compare data from different PCR runs or cDNA samples, Ct values for all genes were normalized to the C_t value of one *ubiquitin* gene (Os03g0234200, except when noted otherwise; Miki et al., 2005) using the equation $R_{0\ GOI} / R_{0\ Norm} = (E)^{Ct\ Norm} / (E)^{Ct\ GOI}$ (*GOI* stands for gene of interest, while *Norm* stands for the internal normalizer gene). Pairs of primers used are listed in Table I.

9. Statistical analysis

Data were subjected to the Student's t-test or to ANOVA followed by comparison of means by the Duncan test ($P \leq 0.05$), except when noted otherwise. All calculations were made using the SPSS Base 10.0 for Windows (SPSS Inc., USA).

Results

1. ZIP mutant lines identification and characterization

In order to evaluate the function of ZIP genes by reverse genetics, a BLAST search was performed against the Rice *Tos17* Insertion Mutant Database using the genomic sequence of all characterized and putative rice ZIP genes. Three mutant lines were found for three different ZIP genes: NE0018 (*zip3*) for *OsZIP3*, NE1520 (*zip4*) for *OsZIP4*, and ND7016 (*zip7*) for *OsZIP7*. The *zip3* line insertion is located at the promoter region 368 bp upstream of the transcription start site (Fig. 1A). The *zip4* line insertion is located at the last exon, 12 bp upstream from the stop codon (Fig. 1A). The *zip7* insertion is located exactly at the boundary between the second intron and the third exon (Fig. 1A). Genes with the insertional mutation had their expression analyzed in seedlings, and both *zip4* and *zip7* lines showed a clear reduction in expression of the respective disrupted genes (Fig. 1B and 1C). No *OsZIP3* transcripts could be detected in roots and leaves of seedlings. However, gene expression analyses are being performed with panicles at the R3 stage, since *OsZIP3* was most expressed in this organ at this stage (data to be included in the manuscript before submission to a journal).

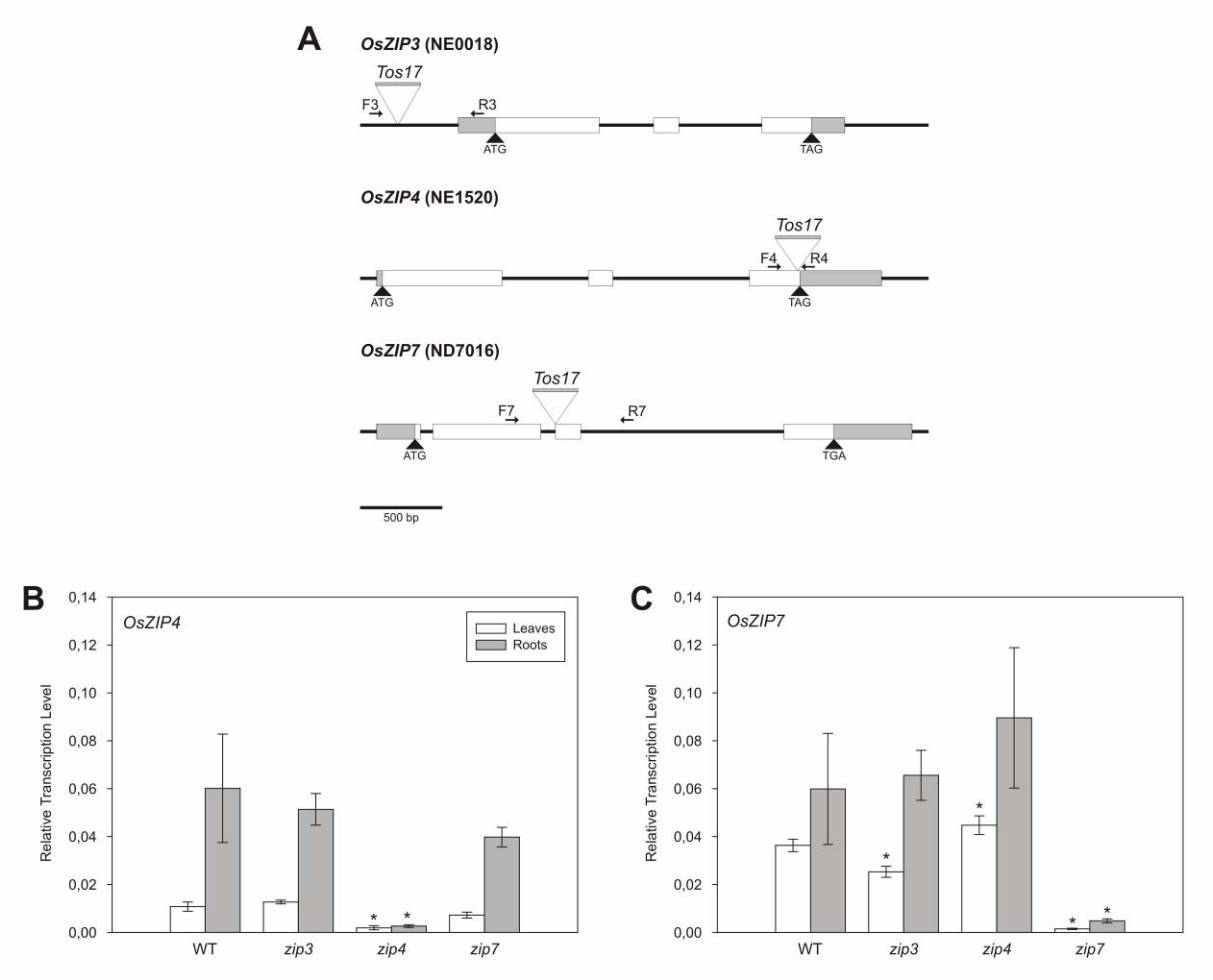

Figure 1. Molecular analysis of *Tos17* mutants. **A.** Schematic representation of *OsZIP3*, *OsZIP4* and *OsZIP7* genes showing exons (gray boxes), introns (black lines), and translational initiation and termination codons (arrowheads). The location of *Tos17* insertions and primers used to identify homozygous plants in *zip3* (F3 and R3), *zip4* (F4 and R4) and *zip7* (F7 and R7) are indicated. **B.** Expression of *OsZIP4* in seedlings. Gene expression was determined for wild type (WT) and mutant seedlings under Zn deprivation for 7 days. The level of expression of each gene is shown relative to *OsUBQ* as an internal reference. Values are mean ± SE of three replicates, and each replicate represents a pool of roots or leaves from four seedlings. The values indicated by an asterisk are statistically different from wild type values according to a t-test ($P < 0.05$). **C.** Same analysis as B, but for the *OsZIP7* gene.

Three-week old seedlings were submitted to Zn sufficiency (2 μM) and deprivation (0 μM) for one week, and after this period no visible phenotypic alterations were detected when comparing wild type and mutant lines (Fig. 2). To check if lines differed in their Zn and other divalent cations concentrations in leaves, three-week old seedlings were submitted for 40 days to three different concentrations of $ZnSO_4$ in the nutrient solution. All genotypes showed a similar pattern of Zn concentration in leaves (Fig. 3A): seedlings were able to achieve similar leaf Zn concentration values when grown under 0.2 and 0.02 μM of $ZnSO_4$, while under 2.0 μM the value was 2.5 times higher in all genotypes. Except for a 2-fold increase in Fe concentration in wild type plants grown under 0.02 μM, there was no consistent pattern for Fe, Mn and Cu leaf concentrations (Fig. 3B-D).

Figure 2. Phenotype of wild type and mutant seedlings. Photograph of four-week old seedlings under Zn deficiency for 7 days. Scale bar = 3 cm.

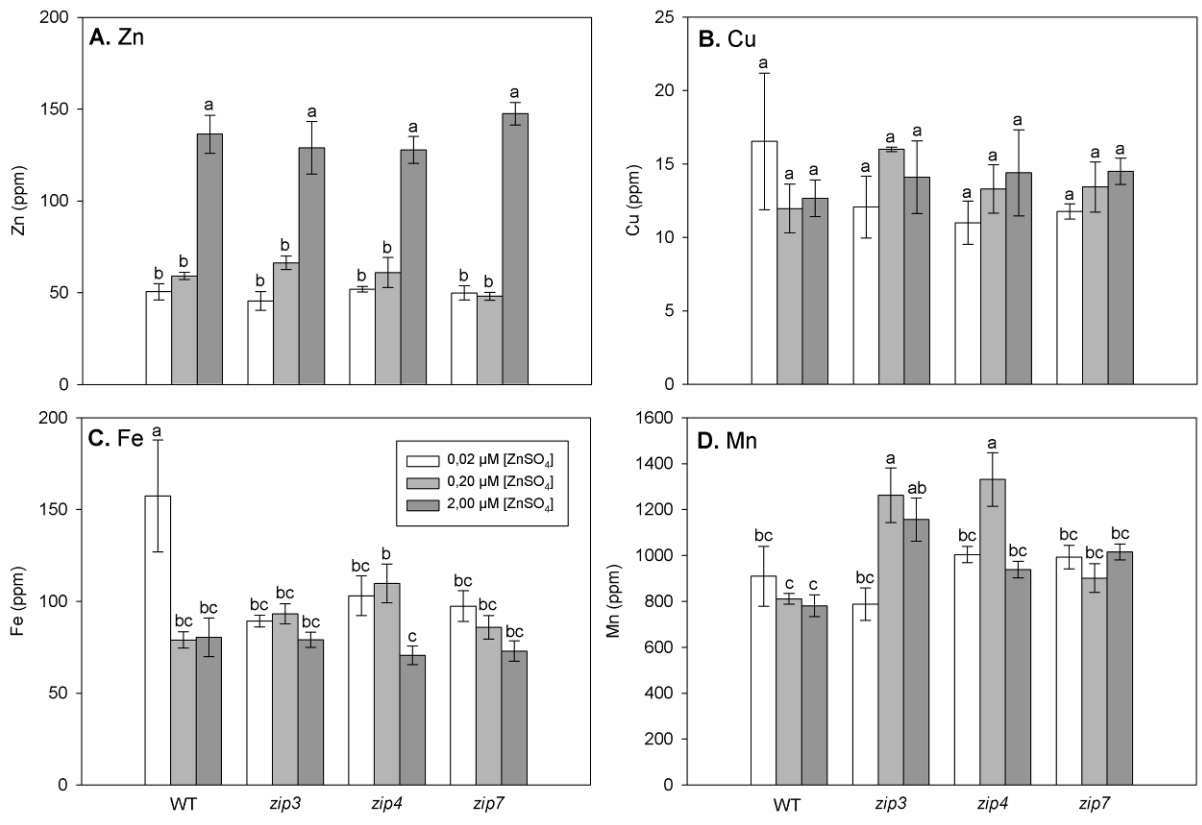

Figure 3. Zinc, copper, iron and manganese concentration in leaves of plants grown for 40 days under three different $ZnSO_4$ concentrations. Values are mean ± SE of three replicates, and each replicate represents a pool of all fully expanded leaves from four seedlings. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

2. Agronomic traits

We observed that *zip3* plants showed a slight delay in reaching the R3 stage under field conditions and this delay was maintained at all subsequent stages when compared to other genotypes. When *zip3* plants reached the R7 stage its phenotype had clearly deviated from that of other lines. Therefore, all plants were allowed to reach the R9 stage (complete panicle maturity) and, to confirm these initial differences, the agronomic traits of the four genotypes were determined (Table 2).

Table 2. Agronomic traits of three *zip* mutants and wild type plants. The values followed by an asterisk are statistically different from wild type values according to a t-test ($P < 0.05$).

Agronomic trait	Wild Type	*zip3*	*zip4*	*zip7*
Plant height (cm)[a]	77.60±1.08	68.40±1.51*	73.00±1.26*	72.20±0.82*
Shoots dry weight (g)[a]	40.79±5.95	53.38±4.21	38.54±1.60	44.97±4.24
Seed yield per plant (g)[a]	51.08±6.70	9.81±1.61*	34.96±1.19	52.67±4.79
Total aboveground biomass (g)[a]	91.88±12.49	63.20±4.92	73.50±2.34	97.64±8.98
Harvest index[a]	55.66±0.95	15.37±2.22*	47.59±1.08*	54.00±0.55
Seed weight (g 1000 seeds^{-1})[a]	27.37±0.38	26.95±0.16	27.02±0.19	27.61±0.16
Panicles per plant[a]	36.20±5.54	71.60±5.85*	35.80±1.82	39.00±3.06 b
Panicle weight (g seeds panicle^{-1})[b]	1.81±0.12	0.86±0.08*	1.97±0.11	2.02±0.15*
Filled spikelets percentage (%)[b]	93.82±0.86	39.04±3.56*	81.36±2.47*	90.75±0.93

[a]Each value represents the mean of five replicate analyses ± standard error.

[b]Each value represents the mean of thirty replicate analyses (six from each plant) ± SE.

All three mutant lines presented lower plant height, especially *zip3* plants which were on average 9 cm shorter than wild type. No statistical difference was detected concerning shoots dry weight and total aboveground biomass, but we call attention to the fact that shoots dry weight from *zip3* plants was on average 30% higher than in wild type plants. Only *zip3* plants showed a significant reduction in seed yield per plant, which might be explained by the evident decrease in filled spikelets percentage. Actually, the unfilled spikelets were noticeable just looking at *zip3* panicles (Fig. 4). The seed individual weight was not significantly different between genotypes according to the weight per 1000 filled seeds. The lower filled spikelets percentage also led to a lower panicle weight and lower harvest index for *zip3*

plants. Even though *zip4* plants similarly had lower harvest index, the reduction was much more severe for *zip3* plants. Interestingly, the number of panicles per plants almost doubled in *zip3* compared to wild type.

Similar results were obtained for two growing seasons at the same location (2007/2008 and 2008/2009 summer season at the UFRGS Faculty of Agronomy, Porto Alegre, RS, Brazil) and for one growing season at two different locations (two experimental fields from the Instituto Rio-Grandense do Arroz, one at Cachoeirinha, RS, Brazil, and another at Penedo, AL, Brazil).

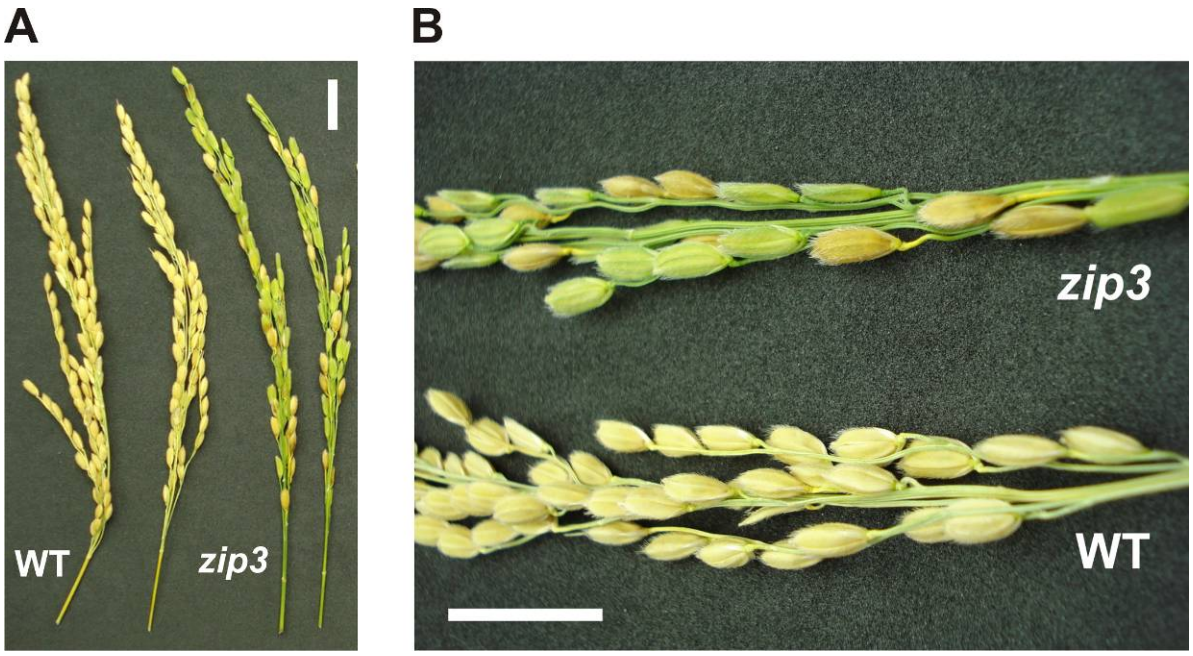

Figure 4. Panicles from mature wild type and *zip3* plants shown in full size (A) and in detail (B). Scale bars = 1,5 cm.

3. Seed metals concentration and panicle metal content

Seeds produced by field-grown plants had their metal concentrations determined and to our surprise Zn and Cu concentrations were higher in *zip3* seeds compared to the other genotypes (Fig. 5A and 5D). Except for a lower Mn concentration of *zip4* seeds, the concentrations of other metals evaluated did not differ (Fig. 5B and 5C). We estimated metal content per panicle after seed metal concentration was multiplied by the spikelets weight for the whole panicle. Even though each individual *zip3* seed contained more Zn and Cu, it became clear that the seed metal content was reduced in *zip3* panicles for all four evaluated metals (Fig. 6A-D).

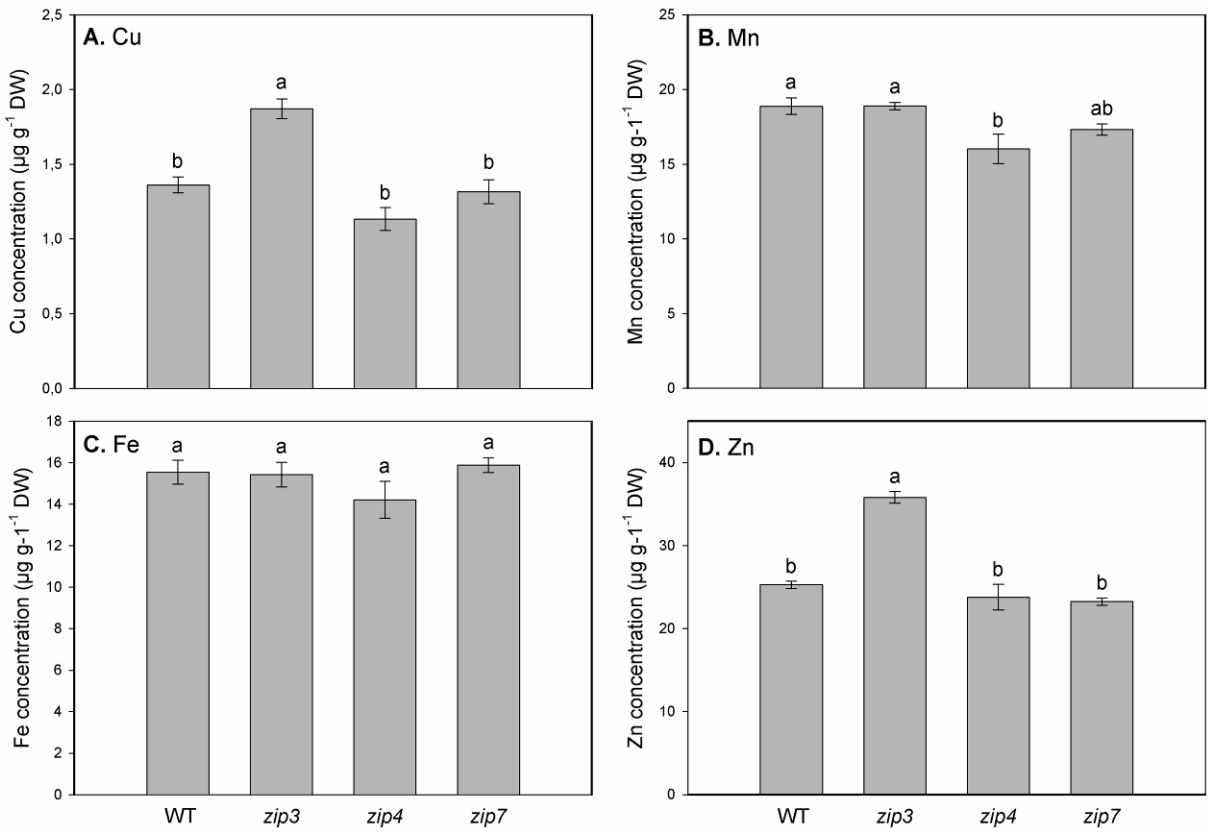

Figure 5. Copper, manganese, iron and zinc concentration in dehusked seeds. Values are mean ± SE of twelve replicates, and each replicate represents a pool of seeds from one panicle. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

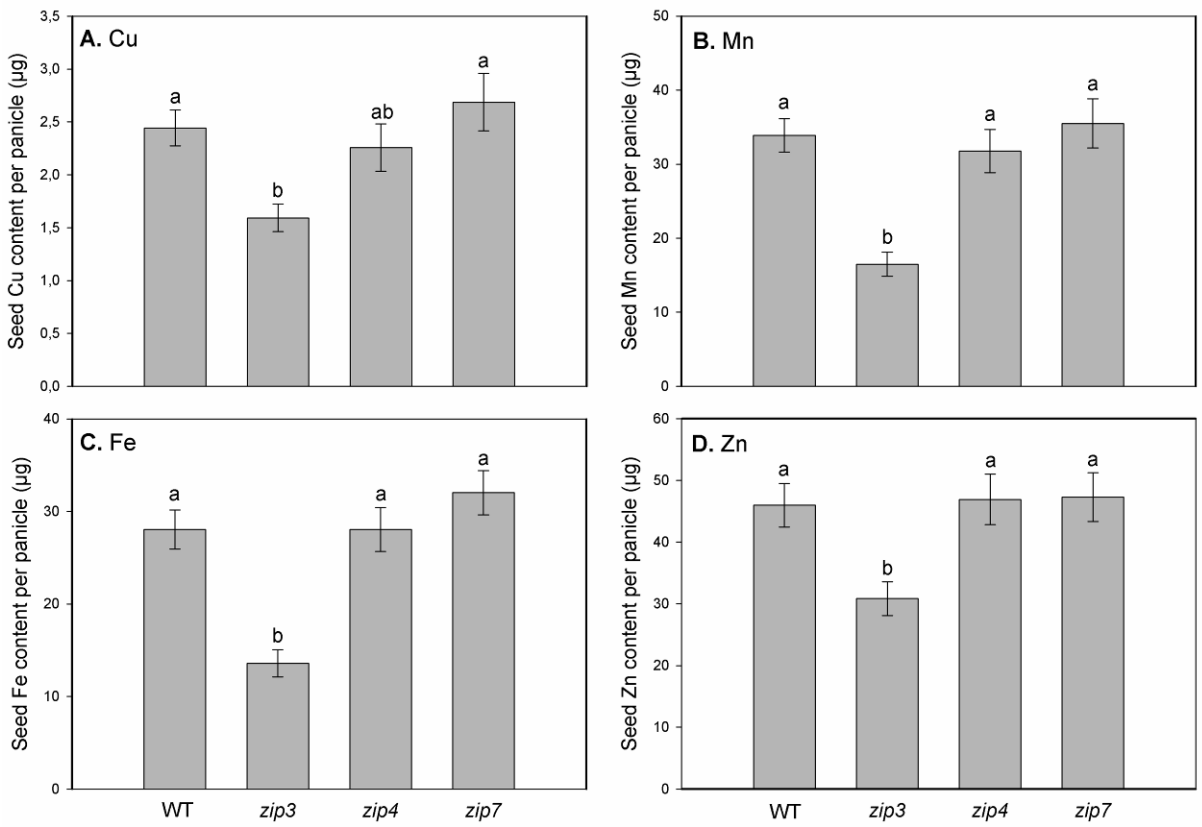

Figure 6. Seed copper, manganese, iron and zinc content per panicle. Values are mean ± SE of twelve replicates, and each replicate represents a pool of seeds from one panicle. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

4. Pollen viability and Zn concentration in *zip3*

Motivated by several studies that assign an essential role for Zn in plant male reproduction (Polar, 1975; Ender et al., 1983; Sharma et al., 1987, Sharma et al., 1990; Pandey et al., 2006), we hypothesized that the low fertility of *zip3* plants could be a consequence of pollen unviability. Wild type and *zip3* pollen grains were collected at R4 stage and their viability was assessed by microscope examination after I_2-KI staining (Fig. 7A). The number of viable pollen grains from *zip3* was half that of wild type, suggesting that pollen unviability is one of the causes to determine *zip3* plants lower fertility (Fig. 7B). Also, the Zn concentration in *zip3* pollen grains was around 48% lower than wild type (Fig. 8A). Interestingly the *Tos17* insertion in the *zip3* mutant only affected the Zn concentration of pollen grains, and not of other different reproductive organs tested (Fig. 8B).

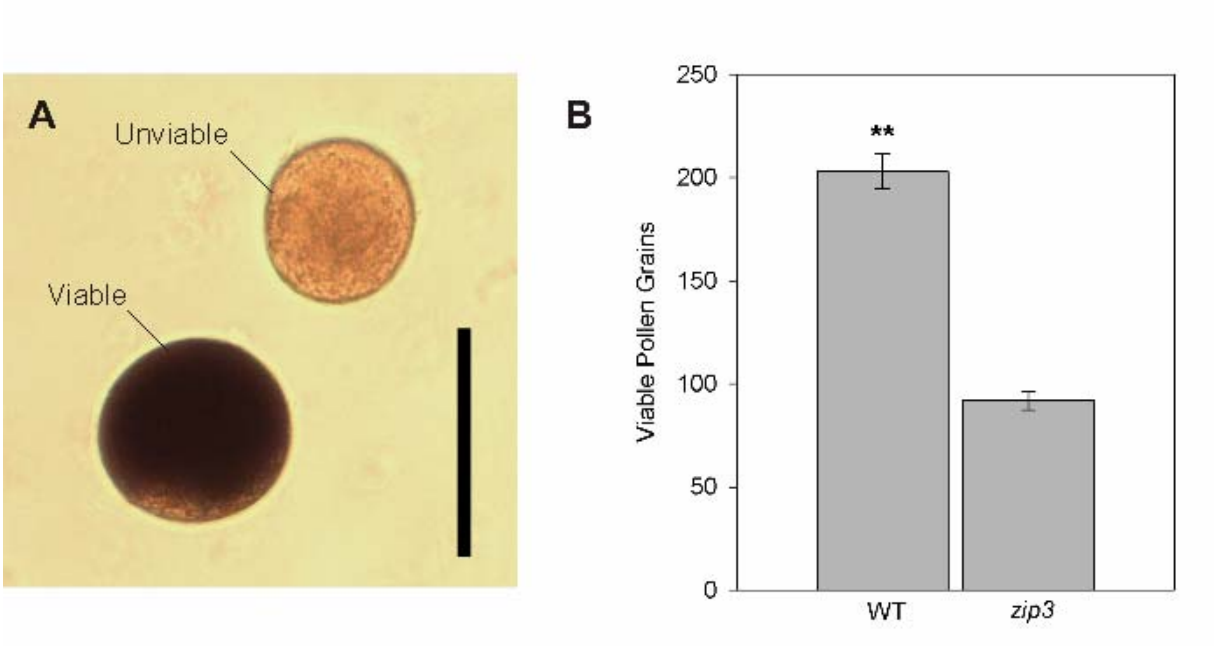

Figure 7. Pollen viability of wild type and *zip3* plants. **A.** Pollen grains stained with 1% I_2-KI. Stained pollen grains were considered viable. Unstained, aborted or small pollen grains were considered unviable. Scale bar = 30 μm. **B.** Pollen viability. Values are mean ± SE of three replicates, and each replicate represents 300 pollen grains evaluated. Statistical difference was determined by t-test (**, $P < 0.01$).

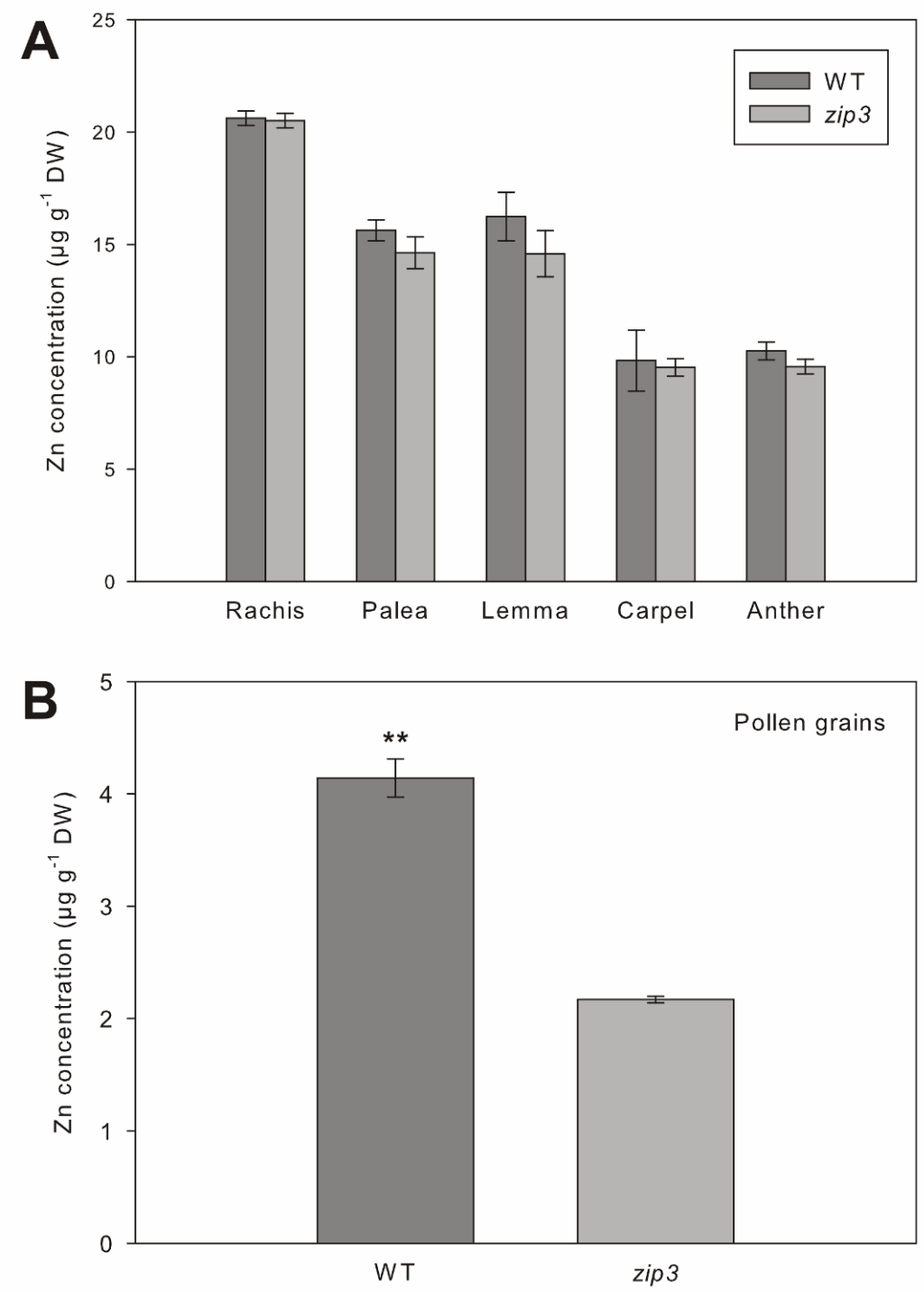

Figure 8. Zinc concentration in reproductive organs and pollen grains. **A.** Zinc concentration in reproductive organs of panicles at R4 stage. Values are mean ± SE of three replicates, and each replicate represents a pool of organs from different florets. No statistical difference was found by t-test. **B.** Zinc concentration in pollen grains. Values are mean ± SE of three replicates, and each replicate represents a pool of pollen grains from different anthers. Statistical difference was determined by t-test (**, $P < 0.001$).

5. Expression in panicles and flag leaves during reproductive stages

Since *zip3* pollen grains showed reduced viability and reduced Zn concentration, we decided to analyze the expression profile of six ZIP genes in panicles and flag leaves at four different reproductive stages: R3 (pre-anthesis), R4 (anthesis), R5 (grain filling) and R7 (grain maturity). No *OsZIP1* transcripts were detected under the conditions tested, and among the other five genes, three of them were more transcribed in flag leaves and two of them in panicles. *OsZIP2* and *OsZIP4* were expressed mainly in flag leaves with a significant induction of *OsZIP2* at R7 stage (Fig. 9A and 9C). *OsZIP4* transcript level was higher at R3 and decreased in the subsequent stages (Fig. 9C). The transcription of *OsZIP7* was kept almost constant during all reproductive stages, except for a lower level in R5 flag leaves and a higher level in R3 panicles (Fig. 9E). *OsZIP3* and *OsZIP5* were more expressed in panicles at R3 with a clear decrease at R4 and very low expression at R5 or R7 stages (Fig. 9B and 9D). Although the pattern was the same, *OsZIP5* transcript levels were always lower than *OsZIP3*.

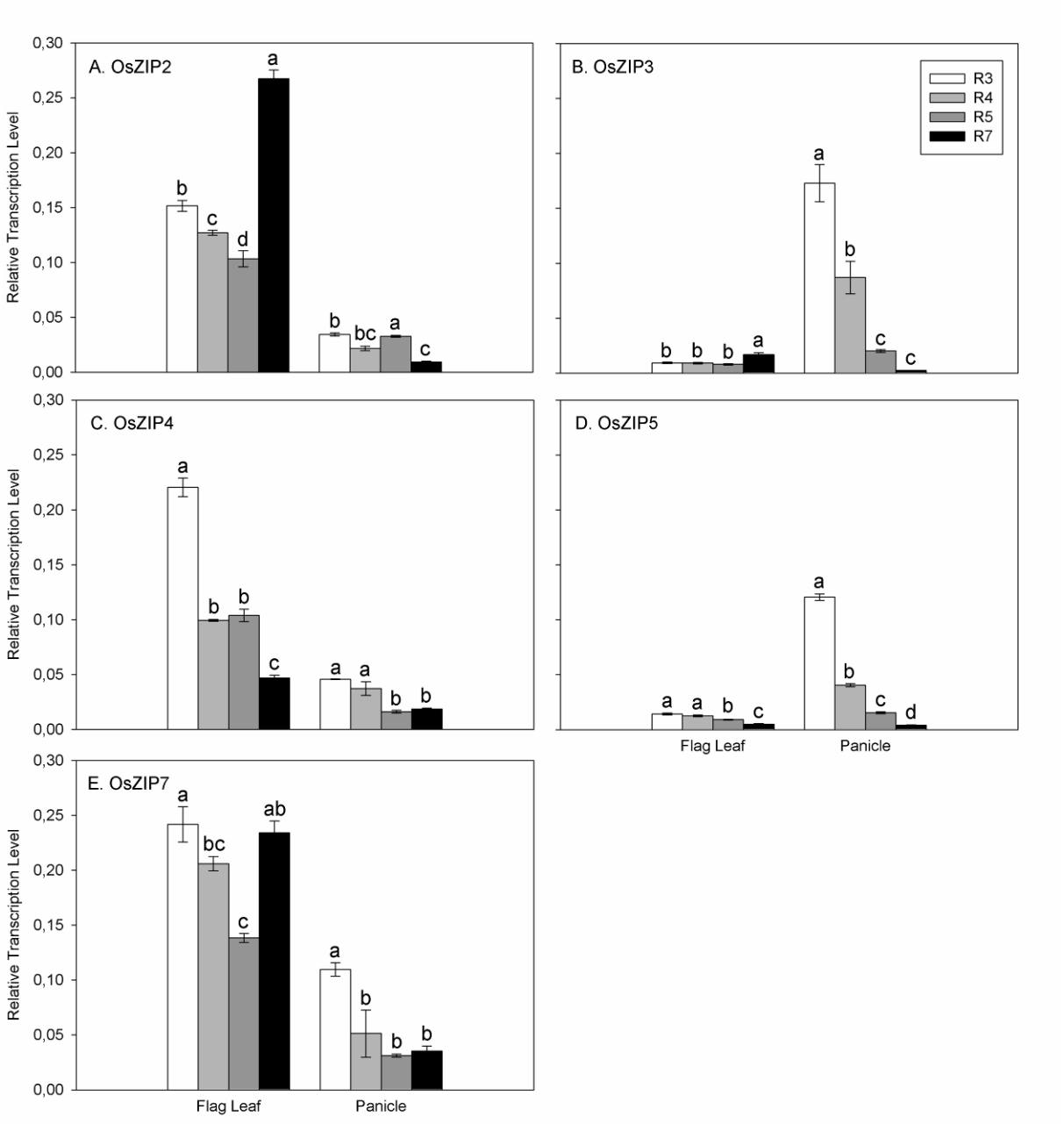

Figure 9. Expression of ZIP family genes in flag leaves and panicles. Gene expression was determined by real-time PCR in wild type field-grown plants. The level of expression of each gene was normalized using an *actin* gene (Os03g0718100; Yokoyama et al., 2004) as an internal standard. Values are mean ± SE of three replicates, and each replicate represents a pool of three panicles or three flag leaves. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

Discussion

Sixteen genes of the ZIP family of metal transporters were identified in the rice genome, and a complete functional characterization of these genes is still lacking. We made use of a reverse genetics approach in order to gain insights into the function *in vivo* of three different ZIP genes (Fig. 1A). Homozygous plants for the *Tos17* insertion lines were isolated and the insertion clearly reduced the transcript levels of the disrupted genes (Fig. 1B and 1C). However, no phenotypic alterations after one week of Zn deficiency could be observed at the seedling stage when comparing with wild type plants (Fig. 2A). Seedlings grown under different Zn concentrations also did not differ in their ability to accumulate Zn in leaves (Fig. 3). Besides a reduction in harvest index and filled spikelets percentage by the *zip4* mutants, mature *zip4* and *zip7* plants also did not develop drastic phenotypic alterations (Table 2). Seeds from these two mutants had Fe, Zn, Mn and Cu concentrations similar to wild type seeds (Fig. 5).

The *OsZIP4* gene had high transcript levels detected in both Zn-deficient roots and shoots, and it complemented a Zn-uptake defective yeast strain (Ishimaru et al., 2005); while *OsZIP7* was induced in Fe-deficient roots and complemented an Fe-uptake defective yeast strain (Yang et al., 2009). Therefore, both of these genes appear to be functional and the lack of phenotypic effects of *zip4* and *zip7* mutations found in our work might suggest a functional redundancy, where the absence of one transporter is compensated by the presence of other transporters. The failure of disruption mutations to produce detectable phenotypic changes is not unusual. For example, it was observed in *Arabidopsis* mutants for metal-chelate transporters of the Yellow Stripe Like (YSL) family: no single mutant had an altered phenotype, and only two of eleven double mutants showed macromolecular phenotypic changes (Curie et al., 2009). The same was observed for *Arabidopsis* mutations on transporters of the Heavy Metal Associated domain proteins (HMA): only the double mutant

hma2hma4 had the Zn translocation to shoots impaired and developed a distinct phenotype (Hussain et al., 2004). Therefore, it would be interesting to evaluate the phenotype of rice double mutants on ZIP family genes in order to check if there is functional redundancy between them. We will also test this possible redundancy by evaluating the expression of non-disrupted ZIP genes in the mutant plants under inductive conditions (data to be included in the manuscript before submission to a journal).

Even though *zip3* plants did not differ visually from wild type plants during vegetative growth, the deviant phenotype of those plants during reproductive stages was noticeable. This pattern should be expected, since we detected *OsZIP3* transcripts mainly in panicles during the R3 stage (panicle exertion) and in lower levels during the R4 stage (anthesis, Fig. 9B). No transcription was detected in roots and leaves, results that are similar to those previously found by quantitative PCR analysis (Ishimaru et al., 2005). However, these results contrast with an earlier Northern blot analysis by Ramesh et al. (2003), who detected OsZIP3 transcripts in both roots and shoots (unfortunately, sequence of probes used is not informed in this study).

Panicles from *zip3* plants had 39% of filled spikelets, compared to 94% of wild type panicles: this led to a 5-fold reduction on total seed yield per plant in the mutant (Table 2 and Fig. 4). Interestingly, *zip3* plants produced on average 30% more vegetative aboveground biomass, which we speculate to be the result of less seeds exerting sink strength over produced assimilates and absorbed minerals; these nutrients were then allocated to vegetative instead of reproductive growth. It was already reported that the removal of all panicles from rice plants led to a two-fold increase in shoots weight (Nakano et al., 1995). Studies with other species also reported that Zn deficiency decreased grain yield even when the effect on shoot biomass yield is marginal (Brennan 1992, Pandey & Sharma, 1998). Additionally, *zip3*

plants doubled the number of panicles per plant compared to wild type plants, as if there was a mechanism to compensate the larger number of unfilled spikelets. In fact, a number of studies indicate that rice plants have adaptations to overcome limitations on grain yield by developing new panicles or increasing the number of spikelets per panicle (Khan & Choudhuri, 1991; Ao et al., 2010). It is worthy to note that rice plants do not seem to rely on increasing individual seed weight to improve grain yield, and *zip3* seeds had similar values for individual seed weight compared to the other genotypes (quantified by the weight per 1000 filled seeds, Table 2). Thus, we propose that the increased vegetative growth and larger number of panicles of *zip3* plants is a consequence of the higher number of unfilled spikelets.

It has been established that Zn is critically required for plant male reproduction, especially because of its function in pollen grains (Polar, 1975; Ender et al., 1983; Sharma et al., 1987, Sharma et al., 1990; Pandey et al., 2006). For example, expression of several zinc-finger proteins has been detected in maize anthers and pollen grains (Ma et al., 2008). Based on that, we speculated that the low fertility of *zip3* plants could be due to pollen unviability. Indeed, the number of viable pollen grains from *zip3* anthers was half of that observed in wild type plants (Fig. 7). To check if pollen unviability was related to Zn deficiency, we determined the concentration of this metal in panicle organs and isolated pollen grains. Panicle organs did not differ on Zn concentration, but *zip3* pollen Zn concentration was about half that of wild type pollen grains (Fig. 8). Considering the expression of *OsZIP3* during panicle exertion and that pollen formation occurs in this stage (Moldenhauer & Gibbons, 2003), we suggest a role for OsZIP3 in Zn transport to the pollen.

Additionally, to our surprise, the seed Zn and Cu concentrations were significantly higher in *zip3* seeds compared to the other genotypes (Fig. 5). However, after we estimated total seed Zn and Cu content per panicle it became evident that even though each individual

seed accumulated more of these metals, each panicle had a reduced content of them (Fig. 6). It seems that the same amount of Zn and Cu reached the panicle during grain filling as in wild type plants, but this amount was distributed between less seeds and this resulted in larger metal concentrations. However, the same scenario would not apply to Mn and Fe (Fig. 5 and Fig. 6).

Here we showed that the insertion of the retrotransposon *Tos17* differentially affected the phenotype of mutants for three rice ZIP genes. While two mutants showed no obvious or detectable phenotype, the *zip3* showed a drastic reduction in the number of filled spikelets and a concomitant increase in vegetative biomass. We propose that disruption of *OsZIP3* impaired Zn loading into pollen grains, what caused lower pollen viability and consequent lower fertility. Further research may elucidate the exact function of *OsZIP3* and the other ZIP genes in the Zn homeostasis network. This information will enhance our understanding of Zn plant nutrition and might support Zn biofortification efforts in the future.

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Capítulo 2:

Sequence analysis and phylogenetic relationships of flowering plants FRO family genes and characterization of the ferric-reductases OsFRO1 and OsFRO2 from rice

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Abstract

Iron (Fe) is one of the essential chemical elements required for plants to complete their life cycles. Iron must be acquired from the soil by root epidermal cells and thereafter translocated to all tissues where it is required, especially green tissues due to its crucial role in photosynthetic processes. Since Fe exists in two interchangeable redox states (Fe^{2+} and Fe^{3+}) inside living organisms, and chelators and transporters show different affinities for these two cationic forms, it has been proposed that reductases from the FRO family might have an important role in Fe homeostasis. This role is probably more proeminent in non-grass species than in grass species. However, the presence of FRO genes in grass genomes indicate that they are also important in this clade. In this study, the phylogenetic relationships of 73 full amino acid sequences of FRO proteins from 21 different flowering plant species were analyzed, focusing in previously characterized functional motifs. It was possible to distinguish three subfamilies of plant FRO proteins, and most of the monocot proteins clustered inside family 2, which has conserved gene architecture and also comprises shoot-expressed genes from *Arabidopsis thaliana*. In order to assess the importance of FRO proteins in grasses, we decided to study OsFRO1 and OsFRO2 from rice by heterologous expression in yeast and phenotypic characterization of two *fro1* mutants.

Introduction

Iron (Fe) is one of the essential chemical elements required for plants to complete their life cycles (Marschner, 1995; Barker & Pilbeam, 2007). Iron exists in two interchangeable redox states inside living organisms (Fe^{2+} and Fe^{3+}), and it readily accepts and donates electrons; Fe also has the ability to form six-coordinate complexes with ligands containing oxygen, nitrogen, and sulfur. Because of these two properties, iron is a critical cofactor of several enzymes involved in diverse metabolic processes, such as the mitochondrial and chloroplast electron transport chain, chlorophyll and ethylene synthesis, and photorespiration. However, these same properties make Fe a particularly problematic micronutrient. First, Fe^{2+} is readily oxidized by atmospheric oxygen and Fe^{3+} shows very low solubility in soils with neutral-to-basic pH values (Hell & Stephan, 2003). Second, free Fe is extremely toxic when present in excessive intracellular levels due to the generation of reactive oxygen species by the Haber-Weiss cycle (Haber & Weiss, 1934; Briat, 2002).

In order to maintain cellular Fe levels inside the narrow range between deficiency and toxicity, plants have evolved a fine-tuned homeostasis network that includes several transporters, enzymes, chelators and storage proteins (recently reviewed in Morrissey & Guerinot, 2009). When growing on Fe-deficient soils, plants employ two different strategies to mobilize insoluble Fe^{3+} (Palmer & Guerinot, 2009). The reduction-based strategy I used by non-grasses involves three coordinated physiological processes at root epidermal cells: (1) proton extrusion by H^+-ATPases to acidify the rhizosphere and turn Fe^{3+} more soluble; (2) reduction of Fe^{3+} by transmembrane ferric-reductases; and (3) absorption of Fe^{2+} by ZIP family transporters (Zinc-regulated, Iron-regulated-like Proteins; Hall & Guerinot, 2006). In contrast, the chelation-based strategy II used by grasses involves two distinct processes: (1) the extrusion of metal chelators known as phytosiderophores (PS) in order to mobilize Fe^{3+},

and (2) absorption of Fe(III)-PS complexes by YSL family transmembrane transporters (Yellow Stripe Like; Curie et al., 2009).

After absorbed from soil, Fe must be kept bound to the appropriate organic molecules in order to avoid undesired reactions inside the cellular environment (Haydon & Cobbett, 2007). Besides metalloproteins binding sites, it is believed that Fe inside cells is chelated by chaperones, ferritin and low molecular weight compounds, such as nicotianamine (von Wirén et al., 1999) and citrate (Abadía et al., 2002). These chelators also are important for long-distance Fe transport through vascular tissues in order to prevent precipitation (Morrissey & Guerinot, 2009). It was proposed that reduction of the metal center promotes the release of Fe from the Fe(III)-PS complexes, and this might be an important step for Fe translocation inside plants (Harrington & Crumbliss, 2009). Furthermore, a study about Fe isotopes fractionation demonstrated that younger tissues from strategy I plants become enriched in light isotopes: an indication of reduction steps along Fe translocation (Guelke & Von Blackenburg, 2007). Therefore, Fe reduction seems to be a critical reaction not only for Fe uptake but also for Fe transport inside the plant body in strategy I plants. Proteins from the FRO family (Ferric Reductase Oxidase) belong to a superfamily of flavocytochromes and have been suggested to function in both processes (Jeong & Connolly, 2009).

The first member of this family to be characterized was AtFRO2 from *Arabidopsis thaliana*, which is the main responsible for the root Fe-reductase activity induced under Fe deficiency stress (Robinson et al., 1999). Other genes encoding FRO proteins from other strategy I species have already been cloned and characterized, namely pea (*Pisum sativum* L.; Waters et al., 2002), tomato (*Solanum lycopersicum* L.; Li et al., 2004), cucumber (*Cucumis sativus* L.; Waters et al., 2007), and peanut (*Arachis hpyogaea* L.; Ding et al., 2009). PsFRO1 and SlFRO1 are expressed in roots and shoots, and four of eight FRO genes from *Arabidopsis* are expressed in shoots (Mukherjee et al., 2006). AtFRO6 proteins localizes to plasma

membrane of green tissue cells (Feng et al., 2006), and AtFRO7 localizes to the chloroplasts of that same tissue (Jeong et al., 2008). Chloroplasts isolated from *atfro7* mutants have decreased ferric-reductase activity and reduced Fe content (Jeong et al., 2008). Additionally, AtFRO3 and AtFRO8 were reported to localize to the mitochondria (Jeong & Connolly, 2009). The first is induced by Fe deficiency in both roots and shoots and its root expression is largely restricted to the vascular cylinder (Mukherjee et al., 2006; Dinneny et al., 2008); the former is highly expressed in senescent leaves, but it is not Fe-regulated (Zimmerman et al., 2004). Thus, it seems that Fe^{3+} reduction is a requirement for leaf cells uptake and organellar transport in *Arabidopsis* as well as in other strategy I species.

In contrast to strategy I plants, species that employ the strategy II for high affinity Fe uptake did not present a fractionation of Fe isotopes (Guelke & Von Blackenburg, 2007). However, at least one strategy II species – rice (*Oryza sativa* L.) – was reported to have putative FRO genes in its genome: *OsFRO1* and *OsFRO2* (Gross et al., 2003). Northern blot analyses allowed the detection of *OsFRO1* transcripts in leaves of Mn-, Zn- and Cu-deficient plants, and *OsFRO2* transcripts were detectable in leaves of Fe-deficient plants (Ishimaru et al., 2006). However, the Fe-reductase activity of these proteins was not assessed and no further functional analysis was performed. In this study, we analyzed the sequence of FRO genes and proteins encoded by putative and functional genes from strategy I and II species. The reductase activity of both rice FRO genes will be evaluated by heterologous expression in yeast. Additionally, we report gene expression analyses to investigate the *in planta* function of FRO rice genes, as well as the phenotypic characterization of two independent rice *fro1* mutants.

Material & Methods

1. Phylogenetic and motif analysis of plant FRO proteins

Deduced amino acid sequences belonging to characterized or hypothetical FRO genes from flowering plants were retrieved from different sources. *Arabidopsis thaliana* sequences were obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org). Rice sequences were also obtained from The Rice Genome Annotation Project (http://rice.plantbiology.msu.edu), The Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp), and OryGenesDB (http://orygenesdb.cirad.fr). Four protein sequences (*Arachis hypogaea*, *Citrus junos*, *Malus xiaojinensis*, and *Pisum sativum*) were retrieved from studies that reported the cloning and characterization of these genes, while the *Tricitum aestivum* FRO protein sequence was obtained from the Triticeae Full-Length CDS DataBase (TriFLDB; http://trifldb.psc.riken.jp/index.pl). All other amino acid sequences were retrieved from Phytozome (http://www.phytozome.net).

All protein sequences obtained were aligned with ClustalX2 (Larkin et al., 2007) and manually checked with BioEdit (Hall et al., 1999). Sequences that displayed significant truncation and/or did not have one or more of the typical functional domains of FRO proteins were discarded. A second alignment was performed with selected sequences and then used to build phylogenetic trees with MEGA4 (Kumar et al., 2008). Conserved motifs were searched using the MEME algorithm with default settings, except that the maximum width was set to 300 amino acids (http://meme.nbcr.net/meme4_1_1/cgi-bin/meme.cgi; Bailey & Elkan, 1995).

2. Plant FRO genes architecture determination

Genomic sequences, cDNA sequences and coding sequences of plant FRO genes were obtained primarily from the same databases as the protein sequence. The cDNA sequences of both rice genes were also retrieved from Rice PIPELINE (http://cdna01.dna.affrc.go.jp/PIPE), while the EST contigs were retrieved from PlantGDB (http://www.plantgdb.org). All obtained sequences were aligned in order to check for inconsistencies. When sequences differed, an alignment with ClustalX2 between the genomic loci, the cDNA and the predicted coding sequence was analyzed. The genomic sequences were given precedence over cDNA clones. After 'consensus' sequences for all genes were obtained, the gene architecture was determined by alignment, including exon-intron boundaries and untranslated regions. The Aramemnon database was queried for predicted transmembrane domains and putative subcellular locations (http://aramemnon.botanik.uni-koeln.de; Schwacke et al., 2003).

3. *Tos17* mutant lines identification and isolation

Search for rice *Tos17* insertion mutant lines was performed in the Rice Genome Resource Center bank (www.rgrc.dna.affrc.go.jp), looking for lines bearing insertions within the regions corresponding to the FRO genes. Seeds of the mutant lines found were requested, and plants were germinated in the laboratory. Genomic DNA of plants from the segregant lines was extracted (Dellaporta et al., 1983). Plants were screened for *Tos17* insertion homozygosis using a PCR-based strategy, with two complementary primers annealing in the genomic region of the gene and another primer annealing in the 3´-*Tos17* tail (Miyao et al., 1998). Amplicons were resolved in 1% agarose gels. All primers used for homozygous identification are indicated in Fig. 5 and their sequences are listed in Table 1. Homozygous plants identified were grown in a greenhouse to provide seeds for the following experiments.

4. Treatments and plant material

Seeds from wild type and identified homozygous mutant plants were surface sterilized in ethanol 70% for 5 minutes, followed by $NaClO_4$ 2.5% for 15 minutes. Seeds were washed with abundant distilled water and germinated in filter paper soaked with water in Petri dishes for four days (at 28ºC, 16 h of light, 40 μmol m^{-2} s^{-1}).

Seedlings were transferred to vermiculite soaked with a nutrient solution containing 0.1 mM KCl, 0.1 mM KH_2PO_4, 0.7 mM K_2SO_4, 2 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 0.5 μM $MnSO_4$, 0.01 μM $(NH_4)_6$ Mo_7 O_{24}, 10 μM H_3BO_3, 0.5 μM $ZnSO_4$, 0.2 μM $CuSO_4$ and 100 μM Fe^{+3}-EDTA. The nutrient solution was buffered with 2 mM MES (2,4-morpholine-ethane sulfonic acid) at pH 5.4, and it was changed every three days. These seedlings were grown for a week and then transferred to 5.0 liter plastic containers containing the same nutrient solution for another week (at 25±2°C, 16 h of light, 160 μmol m^{-2} s^{-1} of light intensity). Plants were then subjected to Fe sufficiency and deficiency (100 μM and 10 μM Fe^{+3}-EDTA respectively) for one week. Leaves and roots from three-week-old plants were separately collected and evaluated for Fe concentration analysis (n = 6), chlorophyll content (n = 6) and gene expression (n = 3).

5. Determination of chlorophyll concentration

Leaves were ground in liquid N_2 and chlorophyll extracted in acetone 85%, quantified by measuring absorbance at 663 and 645 nm (spectrophotometer Cintra 5; GBC Scientific Equipment, Victoria, Australia) and the concentrations of chlorophyll a and b calculated according to Ross (1974).

6. RNA isolation, cDNA synthesis and real-time PCR

Total RNA was isolated using Concert™ (Invitrogen) following the manufactor's instructions. RNA integrity was checked in 1.0% agarose gel, RNA purity was estimated from A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios and quantification performed using Qubit™ (Invitrogen). One microgram of total RNA was treated with RNase-free DNAse I (Invitrogen) and reverse transcribed using M-MLV (Invitrogen) by priming with $Oligo(dT)_{30}$.

Quantitative polymerase chain reactions (qPCR) were performed in an ABI 7500 (Applied Biosystems), using SYBR® Green (Invitrogen) to monitor double strand DNA synthesis. The following standard thermal profile was used for all qPCRs: 95°C for 5 min; 40 cycles of 95°C for 15 sec, 60°C for 10 sec, 72°C for 15 sec, and a final amplification step at 60°C for 35 sec. PCR efficiency values (E) obtained from the exponential phase of each individual amplification plot using the equation $(1 + E) = 10^{slope}$ (Ramakers et al., 2003) were used to normalize each reaction. In order to compare data from different PCR runs or cDNA samples, Ct values for all genes were normalized to the Ct value of the *OsUBQ5* gene (Jain et al., 2006) using the equation $R_{0\ GOI} / R_{0\ Norm} = (E)^{Ct\ Norm} / (E)^{Ct\ GOI}$ (*GOI* stands for gene of interest, while *Norm* stands for the internal normalizer gene). Pairs of primers used for qPCR are listed in Table 1.

Table 1. List of primers used for homozygous identification and quantitative PCR.

Target	Forward (3' → 5')	Reverse (3' → 5')	Amplicon size (bp)
Homozygous identification			
NE8022	GTTTTGCATGGCTTTCCTGT	CAACCAACTGTGGCAACATC	817
NF3020	GATGTTGCCACAGTTGGTTG	GTGATGATGCCCCTCAGTTT	215
NF3035	ATGTTCGTCAAGAACGGGAC	CAACCGCAGGTCAGGTTTAT	525
NF9030	GCAAACTTACCCGGTGTGAT	CAACCAACTGTGGCAACATC	362
Tos17 tail	AGGTTGCAAGTTAGTTAAGA	-	
Quantitative PCR			
OsFRO2	GGGTTACTTGTGTCCCAGTGA	ATCACGATTCTCGTCCCTTG	289
OsSGR	CTACCAAACCGAGCCAAAAT	ACCAAAACGACTCTTGACAGC	170
OsIRO2	CCACAGGAAGCTCAGCCACA	CAGATTCTCCACCTGCTTCTG	178
OsUBQ5	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT	69

7. Addition of recombinational tags

Previously cloned ORFs of OsFRO1, OsFRO2 and PsFRO1 (the last one used as a positive control; Waters et al., 2002) were tagged with vector homology regions (recombination tags, RT) by a two-step PCR. Gene-specific pairs of primers containing half of each RT were designed and used for the primary amplification (Table 2). After the primary amplification, gel band purification was performed with the illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Secondary amplification was performed using the primary amplification products (diluted 1:100) and a pair of primers containing the whole RT (Table 2). The secondary amplification was also purified and further used for recombinational cloning.

8. Recombinational cloning and yeast transformation

Chemically competent *E. coli* KC8 (*pyrF::Tn5 hsdR leuB600 trpC9830 lacΔ4 strA galK hisB436*) were prepared and transformed according to Parrish et al. (2004). The tetracycline-inducible expression vector pCM190 was obtained from the European *Saccharomyces Cerevisiae* Archive for Functional analysis (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/), linearized with enzymes BamHI and NotI, and used for recombinational cloning transformations with the three tagged ORFs. Colonies were screened through colony PCR and plasmids from positive colonies were extracted according to Sambrook et al. (1989). Vectors obtained were sequenced in order to confirm the integrity of cloned ORFs. Wild type strain BY4742 (*MATα his3Δ1 leu2Δ0 lysΔ0 ura3Δ0*; Brachmann et al., 1998) will be transformed according to Gietz & Schiestl (2007).

Table 2. List of primers used for the addition of recombination tags. The sequences relative to the recombination tags are shown in bold.

Name	Forward (3' → 5')	Reverse (3' → 5')
OsFRO1	**TAAATTACCGGATCAATTCGG**ATGACGCCATCCATGATGG	**TAATTACATGATGCGGCCCT**CTACAGGTCGAAGCTGTGGC
OsFRO2	**TAAATTACCGGATCAATTCGG**ATGCGAAATGTTCTTAGGTGG	**TAATTACATGATGCGGCCCT**TCACGCACTCTTCAAAGGTAA
PsFRO1	**TAAATTACCGGATCAATTCGG**ATGGCTCAAGAGAATGTGAAAAG	**TAATTACATGATGCGGCCCT**CTACCAGGTGAAACTGATTGATTC
Secondary amplification	**ACGCAAACACAAATACACACACTAAATTACCGGATCAATTCGG**	**AATGTAAGCGTGACATAACTAATTACATGATGCGGCCCT**

Results & Discussion

1. Protein sequence analysis and conserved motifs

A final set of 73 protein sequences from 21 different species of flowering plants was obtained and used in further analyses: their accession numbers are listed in Table 3, along with their length and a summary of the typical functional domains found in proteins from the FRO family. The length of FRO proteins varied between 528 (OsFRO2) and 824 (BdFRO1) with and average length of 711 amino acids. Even though the genome sequencing of most species included in the analysis is still incomplete, when considering complete as well as partial sequences it is possible to conclude that dicots have a larger number of FRO orthologs (from four in cucumber to eleven in monkey flower) than grasses (two in rice and *Brachypodium* and one in wheat, sorghum and maize). This difference might be a consequence of the inferred greater number of reduction steps during Fe translocation in strategy I compared to strategy II species (Guelke & Blackenburg, 2007).

All FRO proteins characterized until now presented four conserved functional motifs in their sequences – the bis-heme binding motif, the FAD binding motif, the NADPH binding motif and the oxidoreductase signature motif – and all of them were recognizable in the 73 protein sequences evaluated (Table 3 and Fig. 1). A great deal of information about these motifs is derived from studies of mutations observed in the flavocytochrome b_{558} of patients with chronic granulomatous disease in which this molecule is defective (Trasher et al., 1994; Roos et al., 1996).

The bis-heme binding motif comprises four conserved histidines inside transmembrane domains and is involved in heme binding (Finegold et al., 1996). Except for

MgFRO3, all FRO proteins analysed have the four histidines fully conserved (Table 3 and Fig. 1). Amino acids between each pair of histidines are mainly of hydrophobic character, as would be expected of a transmembrane domain. Individual mutation of each histidine to alanine have eliminated all heme binding ability from the yeast FRE1 protein (Finegold et al., 1996), and yeast mutants deficient in heme biosynthesis do not have plasma membrane ferric reductase activity (Lesuisse & Labbe, 1989). These data along with other results obtained for human gp91^{phox} (Taillé et al., 2004) suggest that the presence of the four conserved histidines is crucial for FRO proteins functionality.

The FAD binding motif – defined as HPFT (Yoshida et al., 1998) – showed more variation, especially concerning the last residue: 59% had the canonical threonine, and all others had a serine instead (HPFS; Table 3 and Fig. 1). Additionaly, VvFRO3 was the only protein to have an additional substitution: a serine is found instead of a proline in the second residue (HSPS; Table 4). Different of animal and fungi FRO proteins, all plant proteins analysed also have a fully conserved tryptophan immediately upstream of the characterized FAD binding motif. A substitution of the first histidine for a tryptophan (WPFT) in the human gp91^{phox} reductase was detected in some patients with chronic granulomatous disease and this led to a depletion of FAD in neutrophil membranes, where this protein is localized. Addition of exogenous FAD could not correct that depletion, indicating that this motif is critical for FAD incorporation (Yoshida et al., 1998).

Ishimaru et al. (2006) proposed that the imperfect conservation of the FAD binding motif in OsFRO1 could be indicative that this protein does not have reductase activity. However, it is reasonable to discard this proposition, since 41% of the plant FRO proteins evaluated had the same substitution as OsFRO1, and at least two proteins which have that

very same substitution were demonstrated as having reductase acitivity: AtFRO6 and AtFRO7 (Feng et al., 2006; Jeong et al., 2008).

The NADPH binding motif was much more conserved among evaluated proteins: all of them except three have the consensus GPYG sequence (BdFRO1, MgFRO2, and OsFRO2 have the fourth glycine substituted by a serine: GPYS; Table 3 and Fig. 1). It is not clear if this mutation impairs the NADPH binding ability of FRO proteins, since this motif was identified by structural modeling (Taylor et al., 1993) and no mutant analysis was reported to the best of our knowledge. Finally, the most variable motif analyzed was the oxidoreductase signature motif: it comprises fifteen residues mostly hydrophobic but only nine might be considered conserved sites (the same residue in 50% or more of the sequences; Table 3 and Fig. 1). Despite the variation observed in the remaining six sites, only three of them presented non-conservative substitutions.

Table 3. Plant FRO proteins.

Gene	Source	Size (aa)	FAD	NADPH	Bis-heme	Oxidoreductase signature motif
***Arachis hypogaea* (peanut)**						
AhFRO1	Ding et al. (2009)	728	HPFT	GPYG	+	LVMVSGGSGITPFIS
***Arabidopsis lyrata* (lyrate rockcress)**						
AlFRO1	909813	704	HPFT	GPYG	+	LVMVSGGSGITPFIS
AlFRO2	470119	725	HPFT	GPYG	+	LVMVCGGSGITPFIS
AlFRO3	472552	695	HPFT	GPYG	+	LVMVSGGSGITPFIS
AlFRO4	894281	699	HPFT	GPYG	+	LILVSGGSGITPFIS
AlFRO5	894282	699	HPFT	GPYG	+	LILVGGGSGVTPFIS

Table 3. Plant FRO proteins (cont.).

Gene	Source	Size (aa)	FAD	NADPH	Bis-heme	Oxidoreductase signature motif
AlFRO6	949484	742	HPF**S**	GPYG	+	LVLVAGGIGITPFFA
AlFRO7	949485	741	HPF**S**	GPYG	+	LVLVAGGIGITPFFA
AlFRO8	495072	728	HPF**S**	GPYG	+	LFLVAGGIGITPFLS
AlFRO9	943733	720	HPF**S**	GPYG	+	LVMVSGGSGITPFIS
***Arabidopsis thaliana* (thale cress)**						
AtFRO1	At1g01590	704	HPFT	GPYG	+	LVMVSGASGITPFIS
AtFRO2	At1g01580	725	HPFT	GPYG	+	LVMVCGGSGITPFIS
AtFRO3	At1g23020	693	HPFT	GPYG	+	LVMVSGGSGITPFIS
AtFRO4	At5g23980	704	HPFT	GPYG	+	LILVSGGSGITPFIS
AtFRO5	At5g23990	657	HPFT	GPYG	+	LILVGGGSGVTPFIS
AtFRO6	At5g49730	738	HPF**S**	GPYG	+	LVLVAGGIGITPFFA
AtFRO7	At5g49740	747	HPF**S**	GPYG	+	LVLVAGGIGITPFFA
AtFRO8	At5g50160	728	HPFS	GPYG	+	LFLVAGGIGITPFLS
***Brachypodium distachyon* (purple false brome)**						
BdFRO1	Bradi5g11150.1	824	HPF**S**	GPYG	+	LILVAGGIGISPFLA
BdFRO2	Bradi5g19150.1	735	HPFT	GPY**S**	+	LVMVSGGIGITPFIS
***Carica papaya* (papaya)**						
CpFRO1	evm.model.supercontig_11.38	716	HPFT	GPYG	+	LVMVSGGSGITPFIS
CpFRO2	evm.model.supercontig_11.40	716	HPFT	GPYG	+	LVMVSGGSGITPFIS
CpFRO3	evm.model.supercontig_107.99	668	HPFT	GPYG	+	LIMVSGGSGITPFIS
CpFRO4	evm.model.supercontig_107.98	755	HPF**S**	GPYG	+	LIMISGGSGITPFIS
CpFRO5	evm.model.supercontig_110.3	698	HPF**S**	GPYG	+	LVLVAGGIGITPFLS

Table 3. Plant FRO proteins (cont.).

Gene	Source	Size (aa)	FAD	NADPH	Bis-heme	Oxidoreductase signature motif
Citrus junos **(yuzu)**						
CjFRO1	ABJ16556.1	700	HPFT	GPYG	+	LVMVSGGSGISPFIS
Cucumis sativus **(cucumber)**						
CsFRO1	Cucsa.166120.1	694	HPFT	GPYG	+	LLMISGGSGITPFIS
CsFRO2	Cucsa.260380.1	701	HPF**S**	GPYG	+	LILVAGGIGISPFLA
CsFRO3	Cucsa.108040.1	703	HPFT	GPYG	+	LLLISGGSGITPFIS
Glycine max **(soybean)**						
GmFRO1	Glyma16g03770.1	718	HPFT	GPYG	+	LVMVSGGSGITPFIS
GmFRO2	Glyma18g47060.1	690	HPFT	GPYG	+	IVMVSGGSGITPFIS
GmFRO3	Glyma10g37600.1	702	HPFT	GPYG	+	LVLVSGGSGITPFIS
GmFRO4	Glyma17g09260.1	711	HPF**S**	GPYG	+	LLLVAGGSGITPFLS
GmFRO5	Glyma09g02170.1	734	HPF**S**	GPYG	+	LILVAGGIGLSPFLA
GmFRO6	Glyma15g13090.1	732	HPF**S**	GPYG	+	LILVAGGIGLSPFLA
GmFRO7	Glyma10g37610.1	591	HPFT	GPYG	+	LVLVSGGSGITPFIS
GmFRO8	Glyma05g02600.1	531	HPF**S**	GPYG	+	LLLVAGGSGITPFLS
Manihot esculenta **(cassava)**						
MeFRO1	cassava23164.valid.m1	728	HPF**S**	GPYG	+	LILVAGGIGISPFLA
MeFRO2	cassava40145.m1	747	HPFT	GPYG	+	LVMVSGGSGITPFIS
MeFRO3	cassava43527.valid.m1	707	HPFT	GPYG	+	LVMVSGGSGITPFIS
MeFRO4	cassava21300.m1	718	HPFT	GPYG	+	LVMVSGGSGITPFIS
MeFRO5	cassava7261.valid.m1	692	HPFT	GPYG	+	LVMVGGGSGVTPFIS
MeFRO6	cassava19718.m1	713	HPF**S**	GPYG	+	LVLIAGGIGITPFLS

Table 3. Plant FRO proteins (cont.).

Gene	Source	Size (aa)	FAD	NADPH	Bis-heme	Oxidoreductase signature motif
***Malus xiaojinensis* (apple)**						
MxFRO1	ABU54827.1	721	HPFT	GPYG	+	VVMVSGGSGITPLIS
***Medicago truncatula* (barrel medic)**						
MtFRO1	Medtr1g126580.1	700	HPFT	GPYG	+	IAMVSGGSGITPFIS
MtFRO2	Medtr2g034110.1	740	HPF**S**	GPYG	+	LILVAGGIGLSPFLA
***Mimulus guttatus* (monkey flower)**						
MgFRO1	mg020754m	734	HPFT	GPYG	+	LVMVSGGSGVTPFIS
MgFRO2	mgf022054m	699	HPFT	GPY**S**	+	LVMVSGGSGVTPFIS
MgFRO3	mgf006976m	654	HPF**S**	GPYG	–	LLLVAGGIGITPFLS
***Oryza sativa* (rice)**						
OsFRO1	Os04g0444800	758	HPF**S**	GPYG	+	LILVAGGIGISPFLA
OsFRO2	Os04g0578600	528	HPFT	GPY**S**	+	LVMISGGGGITPFIS
***Pisum sativum* (pea)**						
PsFRO1	AAK95654	712	HPFT	GPYG	+	LVMISGGSGITPFISI
***Populus trichocarpa* (Western poplar)**						
PtFRO1	POPTR_0001s11860.1	734	HPF**S**	GPYG	+	LILVAGGIGISPFLA
PtFRO2	POPTR_0004s07730.1	705	HPFT	GPYG	+	LVLVSGGSGITPFIS
PtFRO3	POPTR_0004s07740.1	689	HPFT	GPYG	+	LVLVSGGSGITPFIS
PtFRO4	POPTR_0012s08670.1	743	HPF**S**	GPYG	+	LLLIAGGAGITPFLS
PtFRO5	POPTR_0015s09480.1	722	HPF**S**	GPYG	+	LLMIAGGAGITPFLS
PtFRO6	POPTR_0017s01690.1	685	HPFT	GPYG	+	LVLVSGGSGITPFIS
PtFRO7	POPTR_0017s01700.1	705	HPFT	GPYG	+	LVLVSGGSGVTPFIS

Table 3. Plant FRO proteins (cont.).

Gene	Source	Size (aa)	FAD	NADPH	Bis-heme	Oxidoreductase signature motif
Ricinus communis **(castor bean plant)**						
RcFRO1	30063.m001432	735	HPF**S**	GPYG	+	LILVAGGIGISPFLA
RcFRO2	30174.m008640	716	HPFT	GPYG	+	LVMVSGGSGITPFIT
RcFRO3	30147.m013879	726	HPF**S**	GPYG	+	LLLIAGGIGITPFLS
RcFRO4	30174.m008639	714	HPFT	GPYG	+	LVMVSGGSGITPFVS
Sorghum bicolor **(sorghum)**						
SbFRO1	Sb06g017800.1	767	HPF**S**	GPYG	+	LILVAGGTGTSPFLA
Solanum lycopersicum **(tomato)**						
SlFRO1	AAT01415	719	HPFT	GPYG	+	LVMISGGSGITPFIS
Triticum aestivum **(common wheat)**						
TaFRO1	tplb0010o07	747	HPF**S**	GPYG	+	LILVAGGIGISPFLAI
Vitis vinifera **(wine grape)**						
VvFRO1	GSVIVT00026928001	715	HPFT	GPYG	+	LVMVSGGSGITPFIS
VvFRO2	GSVIVT00026930001	715	HPFT	GPYG	+	LVMVSGGSGITPFIS
VvFRO3	GSVIVT00015996001	692	H**S**F**S**	GPYG	+	LLLVAGGVGITPFLS
VvFRO4	GSVIVT00011355001	735	HPF**S**	GPYG	+	LVLVAGGIGISPFLA
VvFRO5	GSVIVT00037706001	714	HPFT	GPYG	+	LVMVSGGSGITPFIS
VvFRO6	GSVIVT00037708001	722	HPFT	GPYG	+	LVMVSGGSGVAPFIS
Zea mays **(maize)**						
ZmFRO1	ACG29107.1	760	HPF**S**	GPYG	+	LVLVAGGIGISPFLA

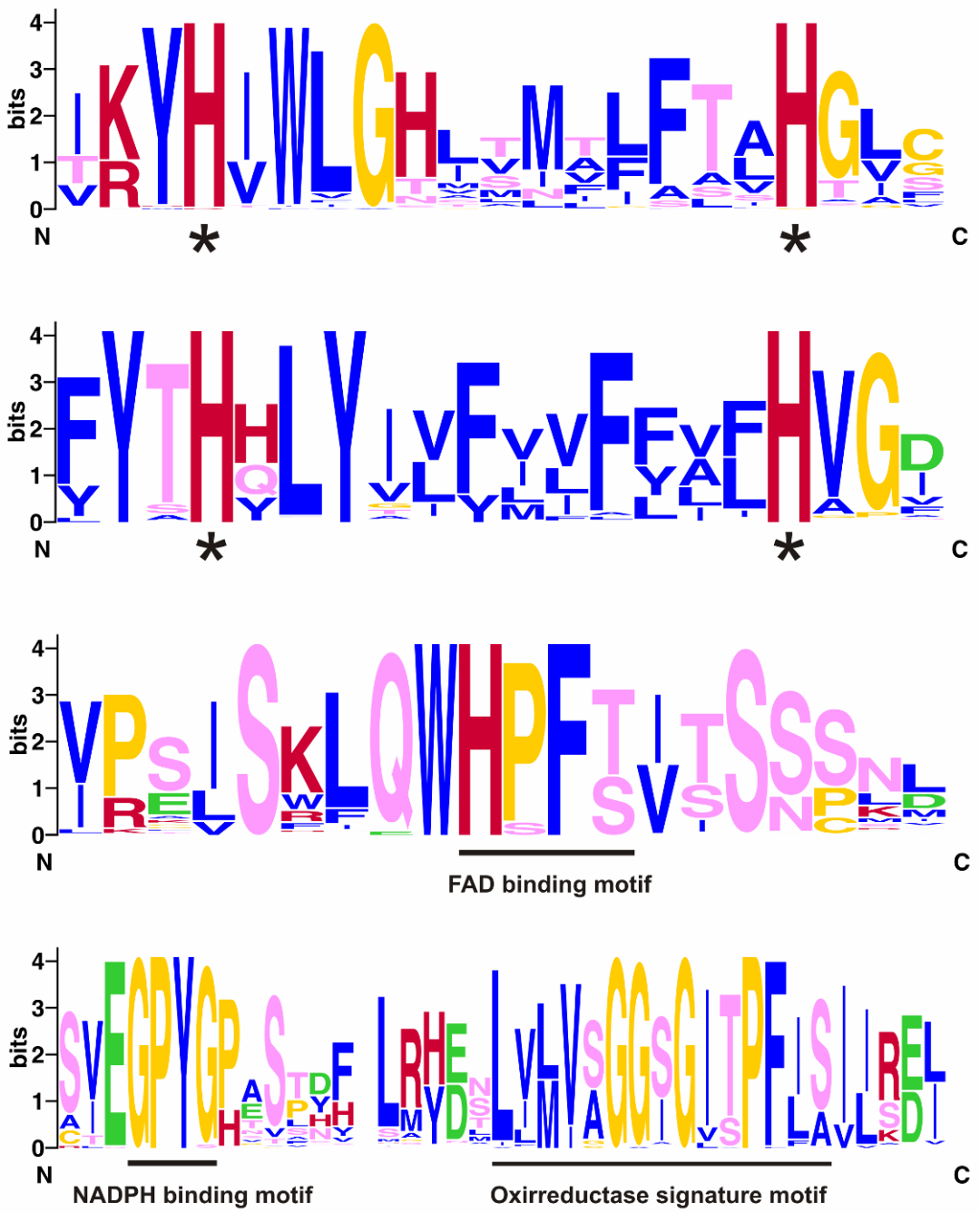

Figure 1. Conservation of typical functional motifs from plant FRO proteins. The conservation of motifs is shown as a WebLogo (Crooks et al., 2004), and it was generated from the alignment of 73 flowering plant FRO protein sequences. Conserved histidines involved in heme binding are indicated by asterisks. Amino acids with similar characteristics are pictured in the same color as follows: positive in red, negative in light green, polar but uncharged in pink, hydrophobic in blue, and special cases in yellow.

2. Phylogenetic analysis

In order to assess sequence similarities in the final set of FRO proteins, a phylogenetic analysis was performed with the Neighbor-joining method on the 73-protein alignment (Fig. 2). Based on tree topology and confidence on nodes (a bootstrap value of 100), we were able to distinguish three clusters which were named subfamilies 1, 2 and 3.

Subfamily 1 included forty-five sequences almost exclusively from dicots: the exceptions are BdFRO2 and OsFRO2. All FRO proteins characterized so far as the main responsible for the root ferric-reductase activity induced by Fe deficiency in strategy I plants clustered inside this subfamily: AtFRO2 (Robinson et al., 1999), PsFRO1 (Waters et al., 2002), SlFRO1 (Li et al., 2004), CsFRO1 (Waters et al., 2007), and AhFRO1 (Ding et al, 2009). *Arabidopsis thaliana* is the species we have most information available about FRO proteins functionality and gene expression, and five of eight proteins from this model organism belong to subfamily 1. These five proteins are expressed in roots (except AtFRO1) as well as in shoots (Mukherjee et al, 2006), and one of them is not localized to the plasma membrane but to the mitochondria (AtFRO3; Jeong & Connoly, 2009). It is also interesting to note that *Arabidosis lyrata*, a closely related species, has nine FRO genes, and the similarity of this additional gene (AlFRO9) to AlFRO2 and AtFRO2 suggests that most likely *Arabidopsis thaliana* have lost one FRO gene after these two species diverged (Fig. 2). OsFRO2 protein appears to have lost a significant N-terminal region when compared to other proteins from this family, and *OsFRO2* transcripts were detected in Fe-deficient shoots (Ishimaru et al., 2006).

Subfamily 3 is composed of eleven sequences and all of them are from dicots. The only member of this family for which we have available information is AtFRO8. It is

localized to the mitochondria, is up-regulated in senescing leaves and it is not regulated by Fe availability (Jeong & Connolly, 2009).

Finally, subfamily 2 comprises seventeen proteins: twelve from dicots and the remaining five from monocots (Fig. 2). Two well characterized FRO proteins from *Arabidopsis thaliana* are found in this family: AtFRO6 and AtFRO7. These proteins are mainly expressed in green tissues: the former is localized to the plasma membrane and the later to the chloroplast (Feng et al., 2006; Jeong et al., 2009). Besides these two proteins, only OsFRO1 was also subject of a published study (Ishimaru et al., 2006): OsFRO1 transcripts were detected in shoots but not in roots, and its expression was induced under Zn-, Mn-, and Cu-deficiency. Consonant with the known fact that strategy II species do not induce root ferric-reductase activity, it was previously reported that these species show negligible fractionation of iron isotopes, which implies that the redox status of Fe does not change during Fe translocation which includes uptake from the soil (Guelke & Blackenburg, 2007). These data, along with the clustering of most monocot sequences inside subfamily 2, led us to the working hypothesis that FRO proteins in grasses function exclusively in leaves and are probably involved in cell or chloroplast Fe uptake.

We also submitted the set of plant FRO proteins to the MEME algorithm in order to find non-functionally characterized conserved motifs, and the eight motifs found in all sequences are schematically depicted in Fig. 2, while their sequences are shown in Fig. 3. It is evident that plant FRO proteins have a core region that is conserved compared to the rest of the protein, and that even FRO proteins of smaller size – such as GmFRO7, GmFRO8 and OsFRO2 – have this core region conserved. This core region comprehends three of the four conserved histidines, as well as the other three typical functional motifs found in FRO proteins.

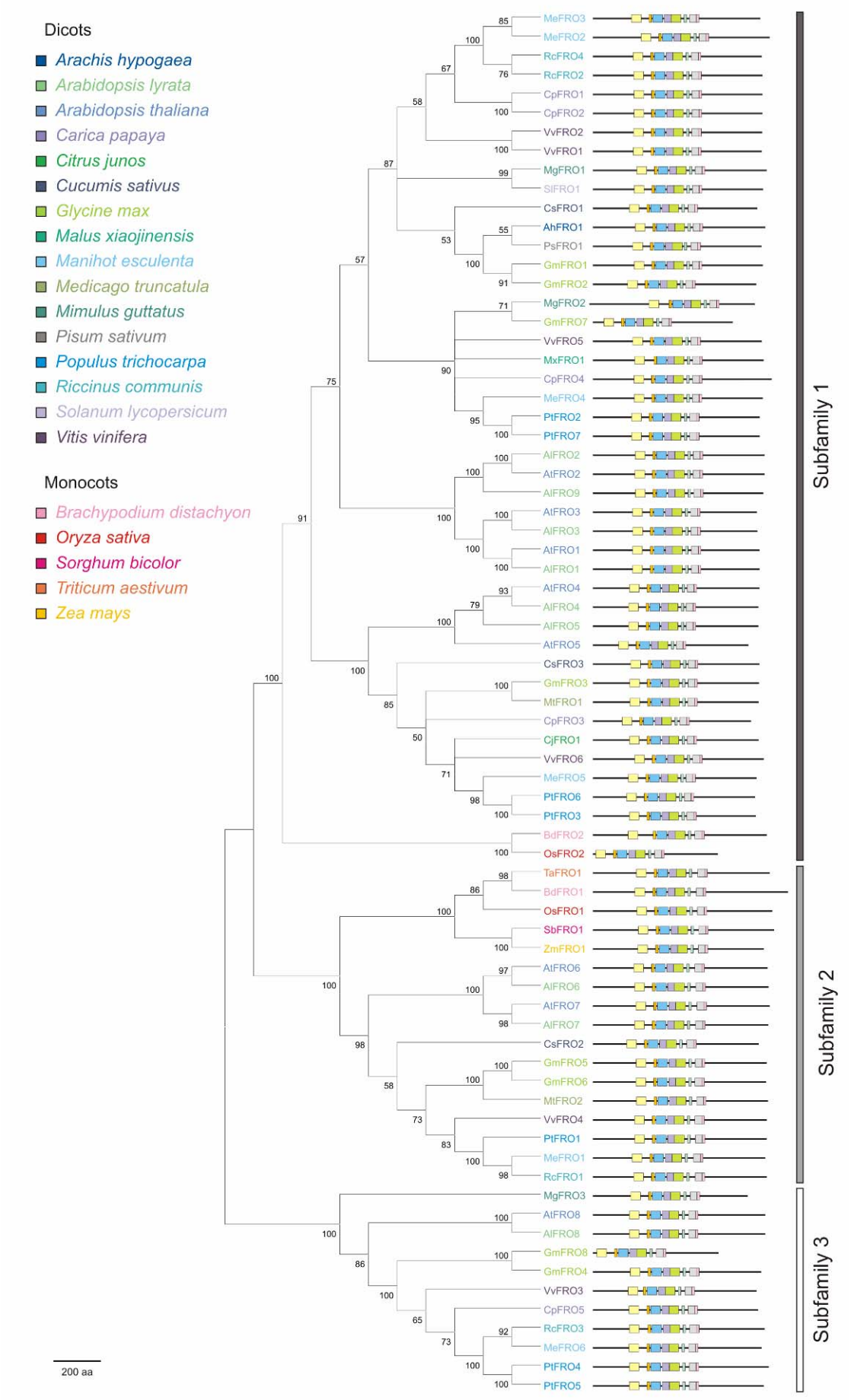
Dicots
Arachis hypogaea
Arabidopsis lyrata
Arabidopsis thaliana
Carica papaya
Citrus junos
Cucumis sativus
Glycine max
Malus xiaojinensis
Manihot esculenta
Medicago truncatula
Mimulus guttatus
Pisum sativum
Populus trichocarpa
Riccinus communis
Solanum lycopersicum
Vitis vinifera
Monocots
Brachypodium distachyon
Oryza sativa
Sorghum bicolor
Triticum aestivum
Zea mays
MeFRO3
MeFRO2
RcFRO4
RcFRO2
CpFRO1
CpFRO2
VvFRO2
VvFRO1
MgFRO1
SlFRO1
CsFRO1
AhFRO1
PsFRO1
GmFRO1
GmFRO2
MgFRO2
GmFRO7
VvFRO5
MxFRO1
CpFRO4
MeFRO4
PtFRO2
PtFRO7
AlFRO2
AtFRO2
AlFRO9
AtFRO3
AlFRO3
AtFRO1
AlFRO1
AtFRO4
AlFRO4
AlFRO5
AtFRO5
CsFRO3
GmFRO3
MtFRO1
CpFRO3
CjFRO1
VvFRO6
MeFRO5
PtFRO6
PtFRO3
BdFRO2
OsFRO2
TaFRO1
BdFRO1
OsFRO1
SbFRO1
ZmFRO1
AtFRO6
AlFRO6
AtFRO7
AlFRO7
CsFRO2
GmFRO5
GmFRO6
MtFRO2
VvFRO4
PtFRO1
MeFRO1
RcFRO1
MgFRO3
AtFRO8
AlFRO8
GmFRO8
GmFRO4
VvFRO3
CpFRO5
RcFRO3
MeFRO6
PtFRO4
PtFRO5
Subfamily 1
Subfamily 2
Subfamily 3
200 aa

Figure 2. Phylogenetic relationships of flowering plants FRO proteins and conserved motifs. The similarity was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl & Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (Pairwise deletion option). There were a total of 1,051 positions in the final dataset. An schematic representation of the amino acid sequences with conserved motifs by the MEME algorithm is pictured beside the protein name. Only motifs that were present in all sequences are shown, and their sequences are illustrated in Fig. 3.

In order to investigate the uniformity of gene architecture from subfamily 2 members, alignments of genomic, cDNA and coding sequences were used to resolve exon-intron boundaries and untranslated regions. It was possible to notice that a nine-exon model is conserved in all genes with full coding sequences, except for BdFRO1 (Fig. 4). Another feature that was distinguishable is that introns on average had a larger size in dicots, specially the introns between third and fourth, and fourth and fifth exon. This pattern does not apply to both species from the genus *Arabidopsis*, as would be expected given the small size of their genomes (Arabidopsis Genome Initiative, 2000).

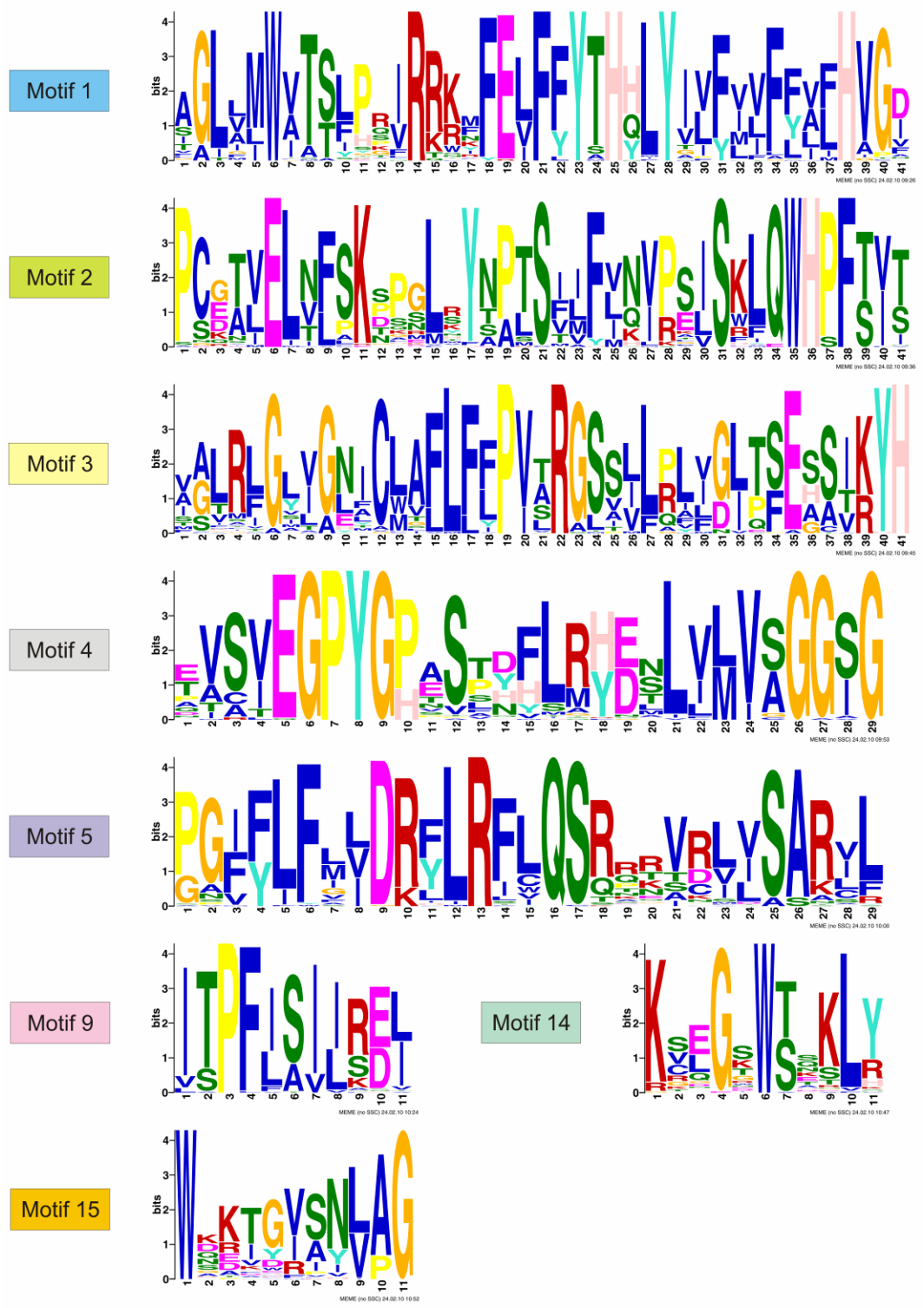

Figure 3. Conserved motifs found by the MEME algorithm in all flowering plant FRO protein sequences evaluated. Motifs are shown as Web Logo and their names are indicated by the same color as they are depicted in Fig. 2.

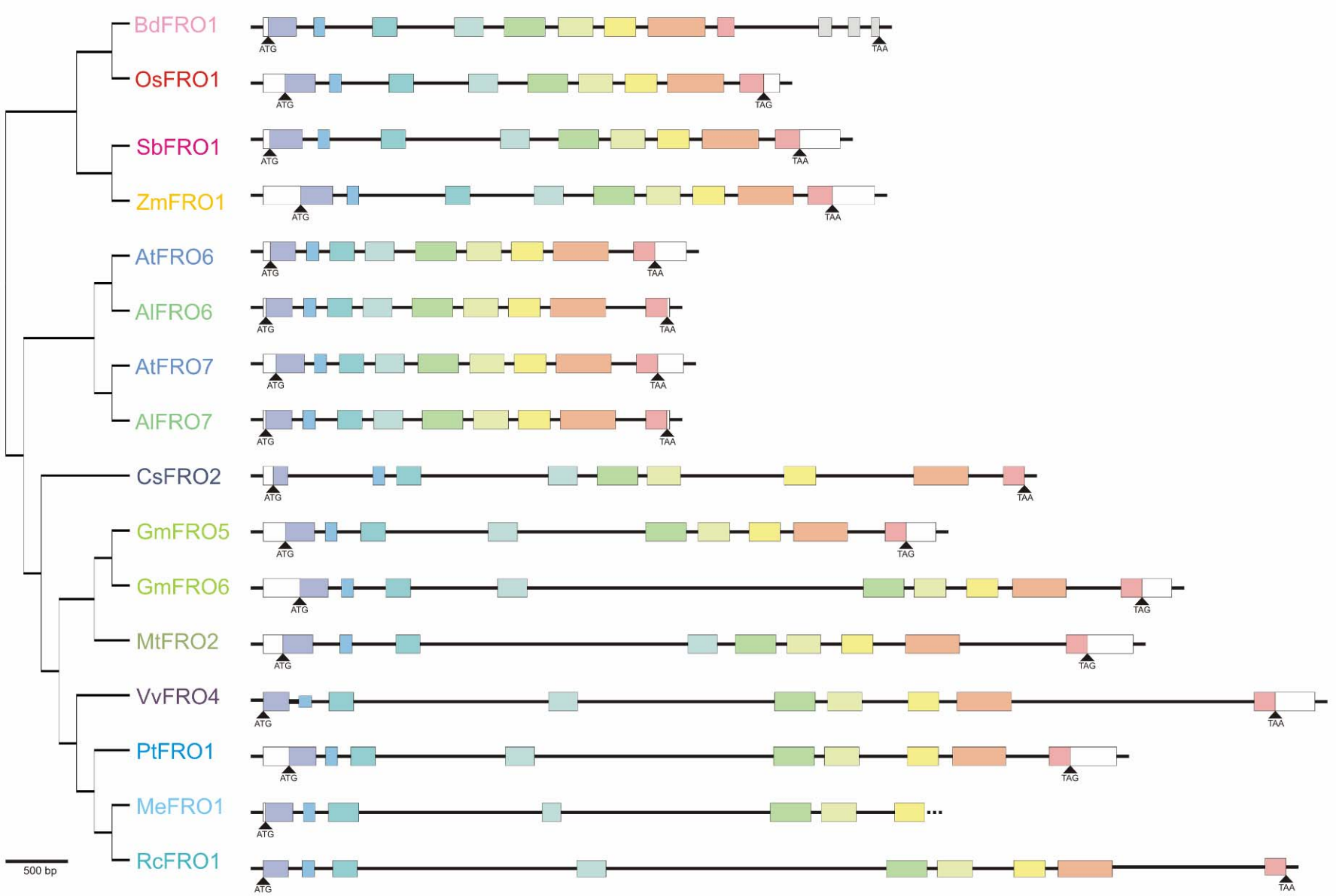
BdFRO1
OsFRO1
SbFRO1
ZmFRO1
AtFRO6
AlFRO6
AtFRO7
AlFRO7
CsFRO2
GmFRO5
GmFRO6
MtFRO2
VvFRO4
PtFRO1
MeFRO1
RcFRO1
ATG
TAA
TAG
500 bp

Figure 4. Gene architecture os flowering plant FRO genes belonging to subfamily 2. Introns are represented as black lines, exons at the same position as boxes of the same color, untranslated regions as white boxes, and translational initiation and termination codons as arrowheads. Genes are clustered according to the phylogenetic analysis illustrated in Fig. 2. TaFRO1 was omitted because no genomic sequence is available. MeFRO1 sequence is partial.

3. Rice FRO genes

In order to analyze the *in vivo* function of FRO proteins in a strategy II species, we decided to study OsFRO1 and OsFRO2 from rice. Both genes are located in the long arm of chromosome 4, and their gene architecture was determined by retrieving genomic, cDNA and predicted coding regions from several sources. The *OsFRO1* gene has the typical nine-exon structure of subfamily 2 FRO genes; the 5' untranslated region comprises 176 bp, the coding region 2,227 bp, and the 3' untranslated region 128 bp (Fig. 5A). In contrast, the *OsFRO2* gene has five exons, and the first two of them are not translated; the 5' untranslated region comprises 721 bp, the coding region 1,587 bp, and the 3' untranslated region 82 bp (Fig. 5B). The *in silico* predicted properties of OsFRO1 and OsFRO2 proteins are summarized in Table 3, and both proteins are predicted to the secretory pathway according to the consensus analysis of the Aramemnon database (Schwacke et al., 2006).

Additionaly, we searched for described cis-elements related to Fe-deficiency responses (Table 4) in the 1,500-bp sequence upstream of the transcription start site. No characterized Fe-related cis-element could be identified in the *OsFRO2* promoter region, but two suggestive alignments were found in the *OsFRO1* equivalent region. First, no significant alignment was found for Iron-Deficiency-responsive Element 1 and 2 (IDE1 and 2) from the barley gene Iron-Deficiency Specific clone n° 2 (IDS2) promoter, which were both necessary for the Fe-deficiency-inducible expression in tobacco roots (Yoshikara et al., 2003; Kobayashi et al., 2003). Second, OsIRO2 is a bHLH transcription factor that regulates genes involved in Fe

uptake under Fe-deficient conditions (Ogo et al., 2006; 2007). It was revealed by cyclic amplification and selection of targets that OsIRO2 preferentially binds to the sequence 5'-ACCACGTGGTTTT-3', while an electrophoretic mobility assay identified 5'-CACGTGG-3' as the core sequence for OsIRO2 binding. Around 800-bp upstream the transcription start site, an alignment of the OsIRO2 binding sequence was found: 5 bp of the 7-bp core sequence were identical plus 2-bp of the 5' portion of the extended OsIRO2 binding sequence. Third, the Iron-Dependent Regulatory Sequence (IDRS) was firstly identified in the promoter region of ferritin genes *AtFER1* and *ZmFER1* and it is responsible for transcriptional repression of *ZmFER1* under low Fe supply conditions (Petit et al., 2001): 11 of 14-bp aligned perfectly with the consensus IDRS in a region approximately 540-bp upstream of the *OsFRO1* transcription start site. These results suggest that *OsFRO1* expression is Fe-regulated, while *OsFRO2* expression is not, in disagreement to a previous Northern blot analysis by Ishimaru et al. (2006). The Northern blot analysis dectected *OsFRO2* transcripts in Fe-deficient leaves and *OsFRO1* transcripts in Zn-, Mn- and Cu-deficient leaves.

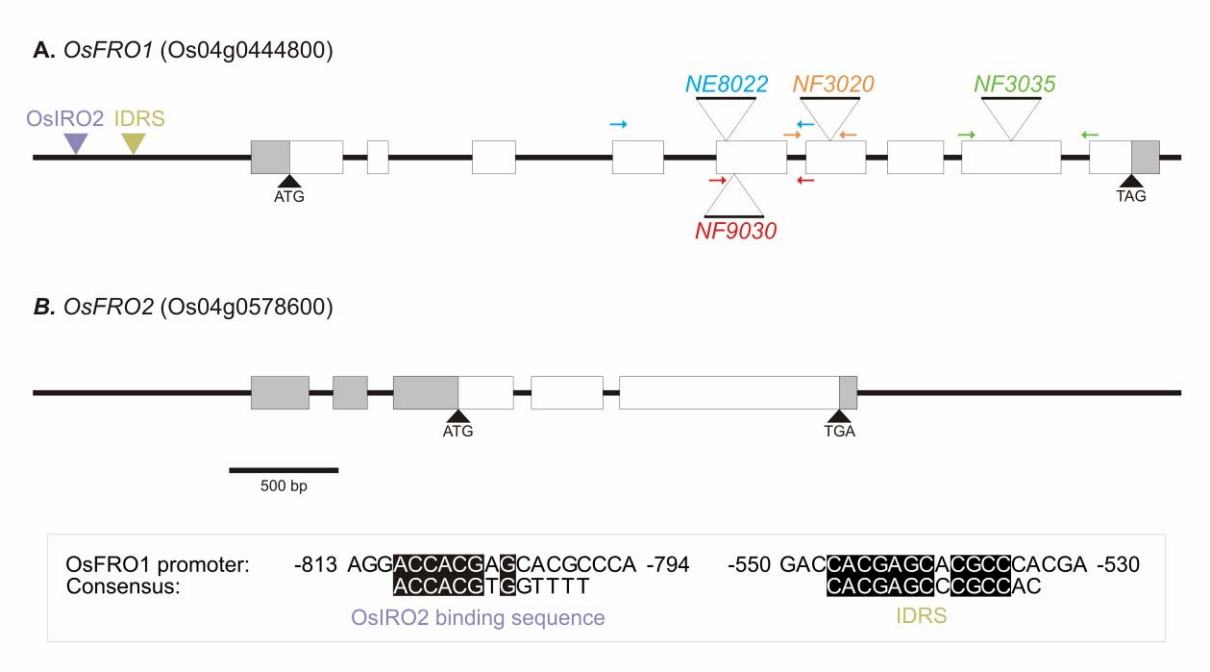

Figure 5. Gene architecture of rice FRO genes. Schematic representation of *OsFRO1* (**A**) and *OsFRO2* (**B**) genes showing exons (white boxes), untranslated regions (gray boxes), introns (black lines), and translational initiation and termination codons (arrowheads). The location of *Tos17* insertions and primers (arrows) used to identify homozygous plants are indicated with the same color. Two Fe-related cis-elements – OsIRO2 binding sequence and IDRS – are indicated in the OsFRO1 promoter region by colored arrowheads, and the alignment is shown inside the box below.

Table 3. Rice FRO genes.

Gene	TIGR Locus	RAP2 Locus	cDNA	TMD	Size (aa)	MW (kDa)	pI	GRAVY
OsFRO1	Os04g36720.1	Os04g0444800	AK070312	10	758	83.17	7.44	0.385
OsFRO2	Os04g48930.4	Os04g0578600	AK068159	7	528	58.48	9.84	0.408

TMD is the predicted number of transmembrane domains (cut-off at 0.5). MW is the predicted molecular weight. pI is the predicted isoelectric point. GRAVY is the grand average of hydropathy.

Table 4. Iron-related cis-elements searched for in both rice FRO genes promoter regions.

cis-element	Sequence	Reference
IDE1 (of HvIDS2)	ATCAAGCATGCTTCTTGC	Kobayashi et al. (2003)
IDE2 (of HvIDS2)	TTGAACGGCAAGTTTCACGCTGTCACT	Kobayashi et al. (2003)
IDRS (consensus)	CACGAGCCCGCCAC	Strozycki et al. (2010)
OsIRO2 binding sequence	AC**CACGTGG**TTTT*	Ogo et al. (2006)

*The core binding sequence for OsIRO2 is shown in bold letters.

4. Homozygous mutants screening and knock-out status

To confirm the suggestions drawn from the *in silico* analysis, we decided to evaluate the function of rice FRO genes by reverse genetics: a BLAST search was performed against the Rice *Tos17* Insertion Mutant Database using the genomic sequence of two previously identified rice FRO genes. Four mutant lines were found for the *OsFRO1* gene: NE8022, NF3020, NF3035 and NF9030. The NE8022 and NF9030 insertions are both localized in the fifth exon; the NF3020 insertion is located in the sixth exon, while the NF3035 is in the eighth exon (Figure 5A). We were able to identify homozygous plants for *Tos17* insertions in two lines: NE8022 and NF3020 (e.g., Fig. 6).

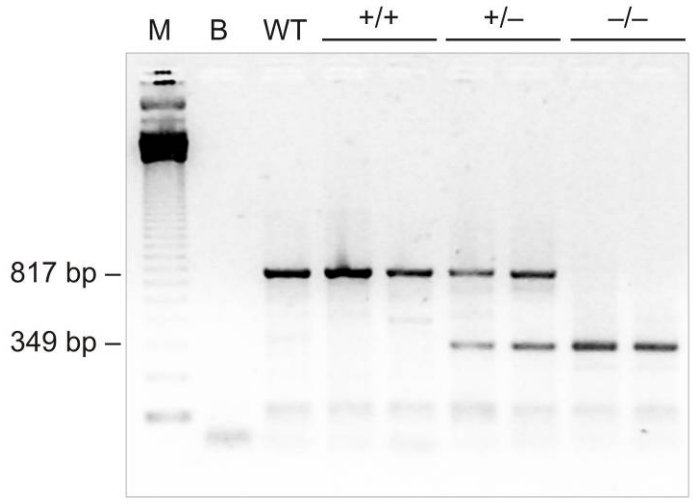

Figure 6. PCR analysis to confirm *Tos17* insertion in the *OsFRO1* gene (NE8022 line). M, molecular mass marker (123 kb plus); B, blank; WT, wild type plant; +/+, homozygous plant without the insertion; +/–, heterozygous plants; –/–, homozygous plants with the insertion.

Mutant plants along wild type plants were supplied with a complete nutrient solution and had the RNA from leaves extracted in order to evaluate the effect of the *Tos17* insertions on *OsFRO1* transcription (Fig. 7). A reverse transcription PCR analysis revealed a band of the expected size only for wild type plants (Fig. 7).

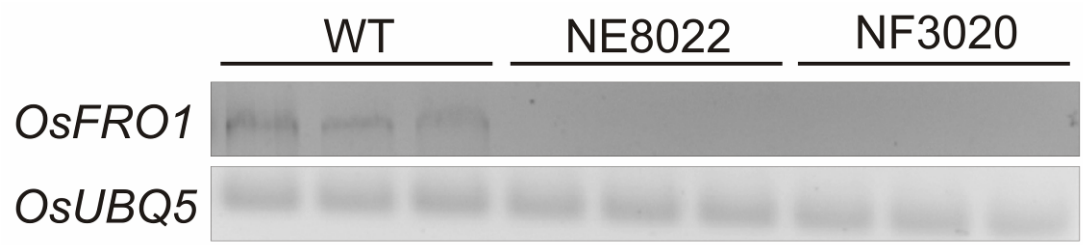

Figure 7. Reverse transcription PCR analysis to confirm the knock-out status of *OsFRO1* in NE8022 and NF3020 lines (45 cycles). The entire open-reading frame of *OsFRO1* was amplified from RNA extracted of leaves. Three plants of each genotype were independently assessed. The *OsUBQ5* gene (40 cycles) was used as an internal control.

5. FRO mutant lines characterization

Wild type and mutant plants grown under Fe sufficiency or deficiency were evaluated for the expression of two genes already characterized as being induced in leaves under Fe deficiency (*Fe-regulated DNA-binding transcription factor, OsIRO2*; Ogo et al., 2007) or senescence (*stay green, OsSGR*; Jiang et al., 2007). According to the significant *OsIRO2* induction in leaves we conclude that plants were actually suffering of Fe deficiency (Fig. 8A), while the low relative transcript levels of *OsSGR* in leaves indicate that these plants were not under a senescence process (Fig 8B).

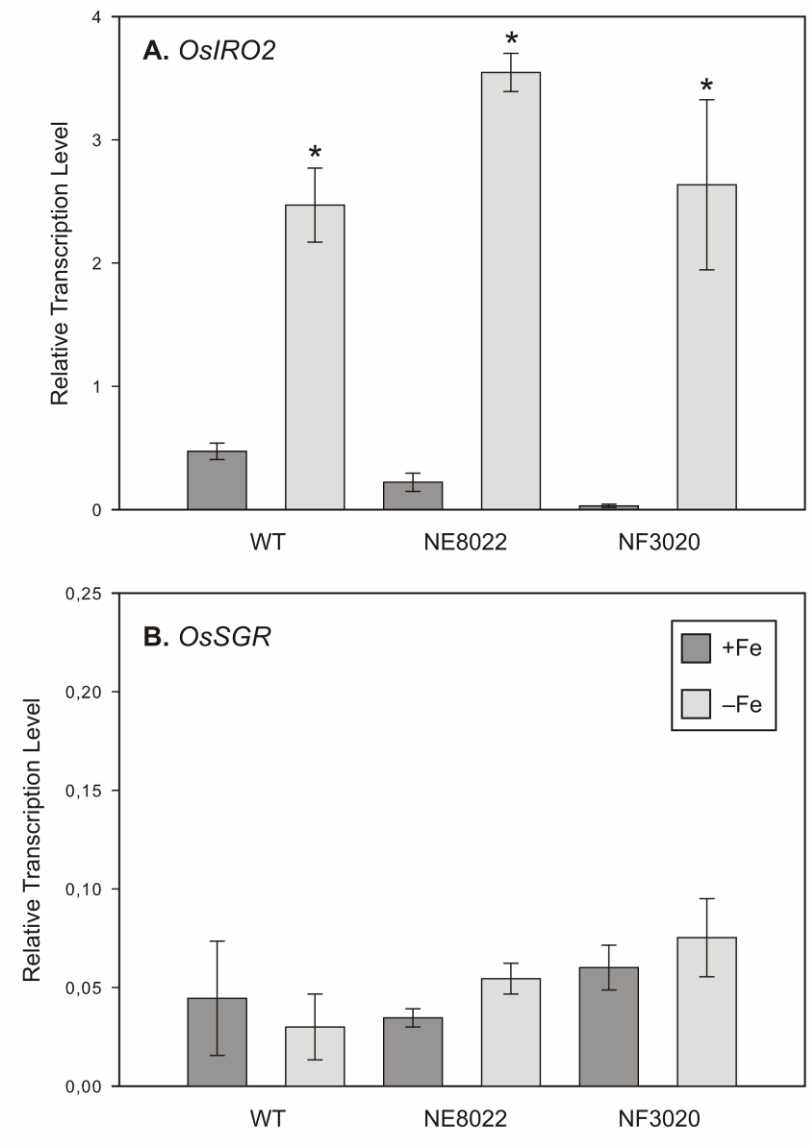

Figure 8. Expression of *OsIRO2* (**A**) and *OsSGR* (**B**) in seedlings' leaves. Gene expression was evaluated for wild type (WT) and mutant seedlings under Fe sufficiency and deficiency for 7 days. The level of expression of each gene is shown relative to *OsUBQ5* as an internal reference. Values are mean ± SE of three replicates, and each replicate represents a pool of leaves from one seedling. The values indicated by an asterisk are statistically different from control (Fe sufficiency) values according to a t-test ($P < 0.05$).

The chlorophyll concentration was reduced by more than 50% in Fe-deficient leaves compared to Fe-suficient leaves (Fig. 9). Interestingly, the chlorosis was more severe in NF3020 leaves compared to the other two genotypes. No *OsFRO2* transcripts could be detected in leaves from plants under control and Fe-deficient conditions. The *OsFRO1* expression was still not assessed in a quantitative manner, since we are having difficulties to obtain a gene-specific and functional primer pair that amplifies a region downstream of the *Tos17* insertion. New pairs of primers will be designed and tested for the further characterization of *fro1* mutants.

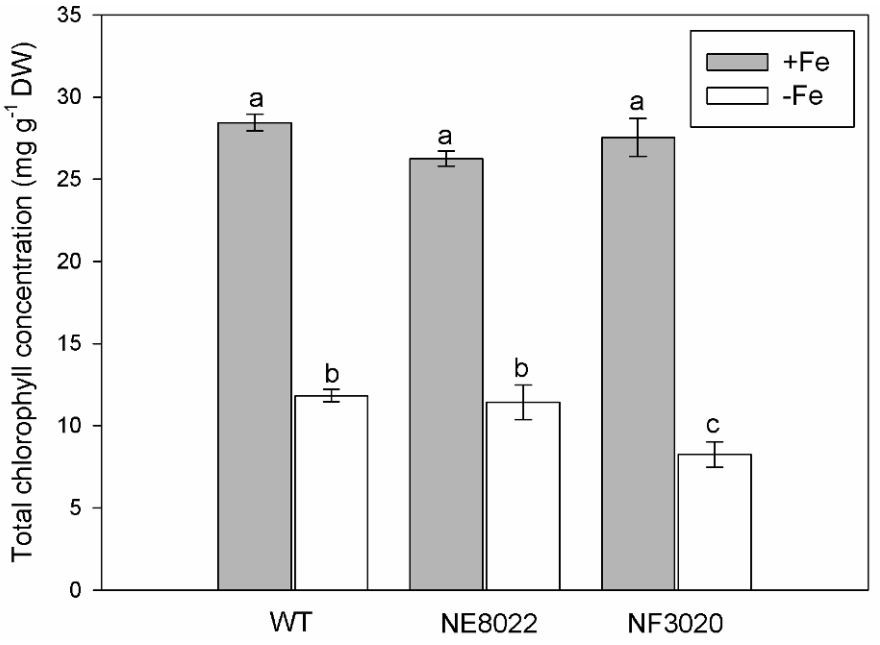

Figure 9. Chlorophyll concentration in leaves from wild type and mutant plants. Values are mean ± SE of six replicates, and each replicate represents a pool of leaves from one plant. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

7. Elemental analyses

Seeds from wild type and mutant plants grown under field conditions (UFRGS Faculty of Agronomy, Porto Alegre, RS, Brazil) were collected and then submitted to elemental analysis by the inductively coupled plasma optical emission spectroscopy (ICP-OES) technique (Fig. 10). Seeds from NF3020 plants had higher concentration for all metals evaluated when compared to wild type seeds (Fig. 10), while NE8022 had higher Zn and Cu concentrations (Fig. 10C-D). New samples of seeds, collected from plants grown in two different locations (experimental fields from the Instituto Rio-Grandense do Arroz at Cachoeirinha, RS, Brazil, and at Penedo, AL, Brazil) are under the same analysis, to confirm the differences seen between the two mutant lines. Such differences in metal concentrations were not expected, since the two lines bear insertions in the same gene. Positional effects of the insertions or other *Tos17* insertions in other positions could be responsible, at least in part, for the different ionomic profile of the two *fro1* insertion mutants.

A similar analysis will be performed with leaves already collected from plants grown in our laboratory that will be sent to the laboratory of Dr. David Salt, at Purdue University, USA.

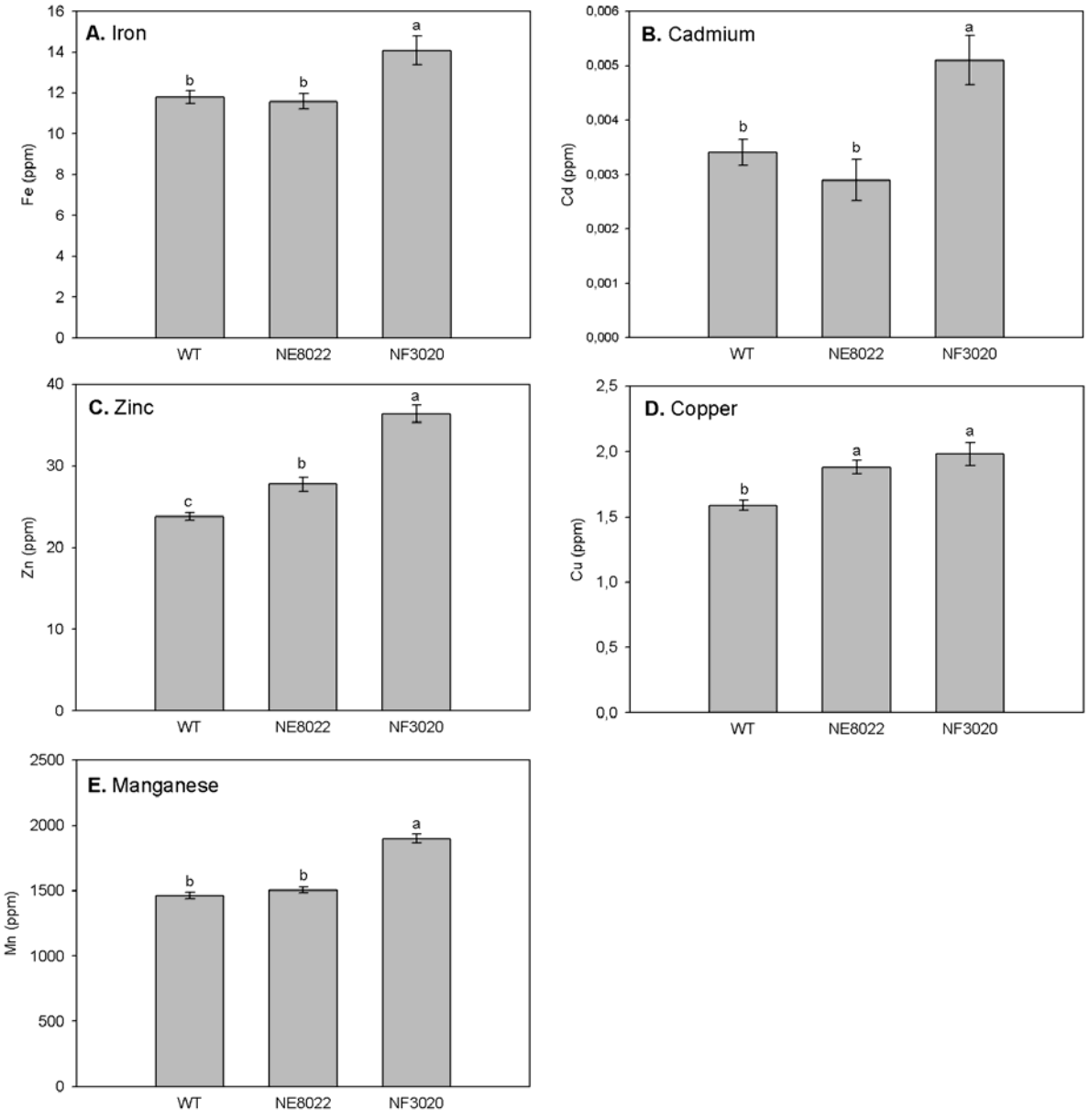

Figure 10. Dehusked seed metal concentrations. Values are mean ± SE of six replicates, and each replicate represents a pool of seeds weighing approximately 250 mg. The values indicated by different letters are statistically different according to a Duncan test ($P < 0.05$).

8. Ferric and cupric reductase activity of rice FRO proteins

There is no report in the literature of an assessment of the ferric and cupric reductase activity of OsFRO1 and OsFRO2 proteins. Therefore, we decided to independently clone the ORFs of these genes into a tetracycline-induced promoter (pCM190) and express both proteins in a wild type yeast strain (BY4742). We have already cloned both genes into this expression vector, along with PsFRO1 from pea, which will be used as a positive control. The yeast transformation protocol is being stablished in our laboratory and will be performed with a wild type strain. The yeast assay approach was already used successfully for other FRO proteins from strategy I species (Waters et al., 2002; Li et al., 2004; Wu et al., 2005).

Most studies have relied on the whole root ferric reductase assay in order to evaluate the *in planta* Fe reduction activity. However, since strategy II plants such as rice do not induce root ferric reductase activity under Fe deficiency (Ishimaru et al., 2006), alternative approaches already described to determine Fe reduction activity in shoots would be to perform a reductase assay with foliar disks (Bruggemann et al, 1993; de la Guardia & Alcantara, 1996; Larbi et al, 2001) or protoplasts (Gonzalez-Vallejo et al., 2000; Siminis & Stravrakakis, 2008; Jeong & Connolly, 2009). It should not be discarded the possibility that rice FRO proteins are localized in a subcellular compartment, which would require an assay using isolated chloroplasts (Jeong et al., 2008) or mitochondria (Jeong & Connolly, 2009), both of which have been reported to be successfully implemented for *Arabidopsis thaliana*.

9. Concluding remarks

We showed that FRO genes and proteins are relatively well conserved among the flowering plant species evaluated. The presence, conservation and expression pattern of FRO genes in rice – a strategy II species – indicate that enzymatic Fe reduction probably take place exclusively in shoots of grasses, which might explain the lack of isotope fractionation previously observed. In order to assess this hypothesis, we began to determine the reductase activity of both rice FRO proteins by heterelogous expression in yeast and to characterize phenotypically two *fro1* mutants.

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Considerações Finais

Os mecanismos envolvidos na homeostase de metais de transição – como o Fe e o Zn – ainda estão longe de estarem completamente elucidados nas plantas. Alguns genes que têm papel importante nesses mecanismos já foram caracterizados para algumas espécies, principalmente em *Arabidopsis thaliana*, arroz e cevada. Porém, muitas famílias que compreendem genes putativos identificados por similaridade de seqüência ainda não foram estudadas com relação aos seus aspectos funcionais.

Por essa razão, resolvemos estudar genes pertencentes a duas dessas famílias – ZIP e FRO – utilizando plantas de arroz com genes interrompidos pelo retrotransposon *Tos17*. Além do seu aspecto científico, por ser uma planta-modelo para o estudo de gramíneas, o arroz também é uma planta economicamente importante, mas pobre em certos nutrientes essenciais para a saúde humana. Portanto, o estudo da homeostase de metais nessa espécie poderá levar a produção de linhagens que apresentem maior valor econômico e/ou um impacto positivo sobre a saúde humana.

No capítulo 1, caracterizou-se fenotipicamente três linhagens mutantes para genes da família ZIP: *zip3*, *zip4* e *zip7*. Enquanto que dois desses mutantes não apresentaram alterações fenotípicas drásticas quando comparados ao tipo selvagem, plantas da linhagem *zip3* tiveram uma clara redução na produção de sementes. Essa redução provavelmente se deve a um maior número de espiguetas vazias, que por sua vez acreditamos que seja conseqüência da menor viabilidade do pólen dessas plantas. Considerando que os grãos de pólen apresentaram menor concentração de Zn e que os maiores níveis de transcritos do gene *OsZIP3* foram detectados em panículas no estágio R3 (quando ocorre a microsporogênese), sugerimos que esse gene tenha um papel no carregamento do Zn para o grão de pólen.

No capítulo 2, realizou-se inicialmente um levantamento das seqüências de genes e proteínas da família FRO de angiospermas. As seqüências obtidas foram analisadas quanto à

presença de motivos funcionais típicos dessa família e comparadas entre si através de análise filogenética. Os resultados obtidos indicam que as proteínas dessa família são relativamente bem conservadas entre as angiospermas, e a maioria delas possui os motivos funcionais analisados. Além disso, a arquitetura gênica também parece ser conservada. Os genes da família FRO podem ser agrupados em três grupos distintos, e um desses grupos – a subfamília 2 – é constituída por genes FRO de dicotiledôneas expressos em parte aérea e também pela maioria dos genes FRO de monocotiledôneas incluídos na análise. A fim de avaliar o papel dos genes FRO em plantas de estratégia II para aquisição de Fe com alta afinidade, iniciamos a caracterização fenotípica de dois mutantes *fro1* por inserção do retrotransposon *Tos17*.

Como perspectiva para atividades futuras, fica pendente a obtenção de alguns dados para a finalização do capítulo 1 em forma de artigo, principalmente a confirmação da diminuição da expressão de *OsZIP3* em panículas no estágio R3, para a qual já temos material coletado. Além disso, ainda resta uma caracterização mais completa dos dois mutantes *fro1* e também avaliar o perfil de expressão dos genes *OsFRO1* e *OsFRO2* em diferentes órgãos e estágios do desenvolvimento. A expressão heteróloga em levedura também deverá fornecer informações sobre a atividade de redutase dessas duas proteínas, e ensaios de localização subcelular poderão auxiliar a compreender o papel exato das proteínas FRO em arroz. Esperamos que a obtenção desses dados permita lançar alguma luz sobre a função *in planta* desses genes e contribuir para uma melhor compreensão da homeostase de Fe e Zn nas plantas de arroz, em particular, e nas gramíneas, em geral.

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