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Identificação de genes importantes para a translocação de Fe e Zn para os grãos de arroz (*Oryza sativa* L.)

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Índice

Lista de abreviaturas

- ABA = ácido abscísico
- ABC = cassette de ligação ao ATP, do inglês *ATP-binding cassette*
- *At = Arabidopsis thaliana*
- BAP = 6-benzylaminopurina
- DMA = ácido deoximuginéico, do inglês *deoxymugineic acid*
- DMAS = ácido deoximuginéico sintase, do inglês *deoxymugineic acid synthase*
- EROs = espécies reactivas de oxigênio
- FER = ferritina
- FPN = ferroportina
- FRO = *ferric reductase oxidase*
- GST1 = glutationa-S-transferase
- HMA = domínio associado a metal pesado, do inglês *heavy metal-associated domain*
- ICP-OES = *inductively-coupled plasma optical emission spectroscopy*
- IDEF = elemento de resposta à deficiência de Fe, do inglês *Fe-deficiency-responsive element factor*
- IRT = transportador regulado por Fe, do inglês *iron-regulated transporter*
- LA-ICP-MS = *laser ablation inductively-coupled plasma mass spectroscopy*
- MES = *2,4-morpholino-ethane sulfonic acid*
- MIR = gene mitochondrial regulado por Fe, do inglês *mitochondrial iron-regulated gene*
- MST = transportador de monossacarídeo, do inglês *monossacharide transporter*
- MTP = proteína de tolerância a metais, do inglês *metal tolerance protein*
- NA = nicotianamina
- NAC = fator de transcrição NAC
- NAS = nicotianamina sintase
- NAAT = nicotianamina amino transferase
- NRAMP = *natural resistance-associated macrophage protein*
- OPT = transportador de oligopeptídeo, do inglês *oligopeptide transporter*
- *Os = Oryza sativa*
- PIC = *permease in chloroplasts*
- PTR = transportador de peptídeo, do inglês *peptide transporter*
- QTL = lócus de características quantitativas, do inglês *quantitative trait loci*

- SAMS = S-adenosil metionina sintetase
- SGR = *staygreen*
- SNAC = proteínas NAC responsivas a stress, do inglês *stress-responsive NAC*
- SSH = hibridização subtrativa supressora, do inglês *suppression subtractive hybridization*
- STP = proteína transportadora de açúcar, do inglês *sugar transport protein*
- VIT = transportador de ferro vacuolar, do inglês *vacuolar iron transporter*
- YSL = *yellow stripe-like*
- ZIFL = *zinc-induced facilitator*
- ZIP = *Zrt/ Irt-like Protein*

Resumo

O arroz é o alimento base para metade da população mundial. Entretanto, é uma fonte pobre em micronutrientes essenciais como Fe e Zn, que são removidos durante o processamento do grão para produção de alimento. Por essa razão, populações cujas dietas baseiam-se principalmente em arroz estão sujeitas à deficiência de Fe e Zn, que são as deficiências nutricionais mais comuns no mundo, afetando mais de dois bilhões de pessoas. Uma vez que as folhas-bandeira são uma das fontes de remobilização de metais para os grãos em desenvolvimento, a identificação dos mecanismos moleculares que contribuem no processo de transporte de metais das folhas-bandeira para os grãos pode ser útil em programas de biofortificação. Dessa forma, utilizamos duas abordagens diferentes para estudar esta questão.

Primeiro, realizamos uma análise por hibridização subtrativa supressora (SSH) em folhas-bandeira de duas cultivares de arroz e isolamos 78 sequências induzidas no estágio de enchimento do grão (R5) em relação ao estágio de emergência da panícula (R3). A expressão diferencial de alguns genes selecionados (entre eles do fator de transcrição *OsNAC5*) foi confirmada por PCR quantitativo. Mostramos que a expressão de *OsNAC5* é ativada em processos de senescência natural (envelhecimento) e induzida (escuro, aplicação de ABA, alta salinidade, frio e deficiência de Fe) e sua expressão não é afetada na presença de 6-benzilaminopurina (um inibidor de senescência) em condição de senescência induzida por escuro. A indução da expressão de *OsNAC5* sob alta salinidade é abolida por nicotinamida, um inibidor dos efeitos do ABA. Este resultado e a presença de elementos *cis*-atuantes na região promotora do gene *OsNAC5* sugere uma regulação dependente de ABA. Utilizando quatro diferentes cultivares de arroz, mostramos que a indução de *OsNAC5* é maior e antecipada em folhas-bandeira e panículas de plantas da cultivar IR75862, que possui as maiores concentrações de Fe, Zn e proteínas nas sementes. Dessa forma, sugerimos que *OsNAC5* é um fator de transcrição do tipo NAC dependente de ABA e associado à senescência, e sua função pode estar relacionada à remobilização de Fe, Zn e aminoácidos dos tecidos verdes para os grãos.

Posteriormente, analisamos a expressão de 25 genes de arroz relacionados à homeostase de metais em folhas-bandeira de oito cultivares de arroz (com níveis contrastantes de Fe e Zn nos grãos) durante os estágios de emergência da panícula (R3) e enchimento do grão (R5). Nove destes genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* e *OsNAC5*) apresentaram correlação significativa entre os níveis de expressão em folhas-bandeira e as concentrações de Fe e Zn nas sementes. Assim, nosso estudo forneceu uma pequena lista de possíveis genes-alvo para manipulação da concentração de Fe e Zn em grãos de arroz.

Abstract

Rice is the staple food for half of the world population. However, it is a poor source of essential micronutrients such as Fe and Zn, most of which are lost during grain processing for food production. For this reason, populations with monotonous diets consisting mainly of rice are especially prone to Fe and Zn deficiency, which are the most common and widespread nutritional disorders in the world, affecting more than 2 billion people. Since flag leaves are one of the sources of remobilized metals for developing seeds, the identification of the molecular players that might contribute to the process of metal transport from flag leaves to the seeds may be useful for biofortification purposes. In this way, we used two different appraches to address this issue.

First, we conducted suppression subtractive hybridization (SSH) analysis in flag leaves of two rice cultivars and isolated 78 sequences up-regulated in flag leaves at the grain filling stage (R5) relative to the panicle exertion stage (R3). Differential expression of selected genes (including the *OsNAC5* transcription factor) was confirmed by quantitative RT-PCR. We showed that *OsNAC5* expression is up-regulated by natural (aging) and induced senescence processes (dark, ABA application, high salinity, cold and Fe-deficiency) and its expression is not affected in the presence of 6-benzylaminopurine (a senescence inhibitor) under dark-induced senescence. Salt induction of *OsNAC5* expression is abolished by nicotinamide, an inhibitor of ABA effects. This result and the presence of c*is*-acting elements in the promoter region of the *OsNAC5* gene suggest an ABA-dependent regulation. Using four different rice cultivars, we showed that *OsNAC5* up-regulation is higher and earlier in flag leaves and panicles of IR75862 plants, which have higher seed concentrations of Fe, Zn and protein. We suggest that *OsNAC5* is a novel senescence-associated ABA-dependent NAC transcription factor and its function could be related to Fe, Zn and amino acids remobilization from green tissues to seeds.

We also analyzed the expression of 25 metal-related genes from rice in flag leaves of eight rice cultivars (showing contrasting levels of seed Fe and Zn) during panicle emergence (R3) and grain filling stage (R5). The expression level of nine of these genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) in flag leaves exhibited significant correlations with Fe and/or Zn concentrations in the seeds. In this way, our study has provided a short list of putative target genes to manipulate Fe and Zn concentrations in rice grains.

Introdução Geral

Importância econômica e científica do arroz

O arroz cultivado, *Oryza sativa* L., pertence à família Poaceae. É considerado uma planta anual semi-aquática, e geralmente cresce até cerca de 1,2 m de altura. Seu fruto, chamado grão ou cariopse, serve de alimento básico para mais da metade da população mundial, principalmente em países em desenvolvimento (Sasaki e Burr, 2000). O arroz é uma espécie importante tanto economicamente, quanto cientificamente. 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), o que faz do arroz a terceira maior cultura de grãos ao redor do mundo, perdendo apenas para milho e trigo. 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). É também a planta modelo para estudos de fisiologia e genética em monocotiledôneas (Shimamoto e Kyozuka, 2001).

No Brasil, a produção é de aproximadamente 13 milhões de toneladas anuais, o que faz do Brasil o décimo maior produtor mundial, sendo o maior produtor não-asiático deste cereal. O consumo médio no Brasil é de aproximadamente 50 a 60 kg anuais por pessoa (http://www.fao.org/rice2004/en/p1.htm). A agricultura do arroz tem um importante papel na economia do Rio Grande do Sul, por ser a principal atividade econômica em inúmeros municípios do estado, principalmente na metade sul. O RS é o estado que mais produz arroz no Brasil, sendo cultivados anualmente em torno de um milhão de hectares com arroz irrigado, os quais são responsáveis por 62,2% da produção nacional desta cultura (IBGE, 2010).

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, o qual é rico em amido e altamente calórico, 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 e Broadley, 2009).

Características gerais do Ferro

O ferro (Fe) é o metal de transição mais abundante nos organismos vivos (Epstein, 1965) e apresenta duas características químicas que explicam em grande parte a sua presença numa ampla gama de sítios ativos de metaloproteínas (Barker e 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). Em sistemas aeróbicos, o Fe ligado a quelantes de baixo peso molecular ou em sua forma livre (Fe^{+3} ou Fe^{+2}) é muito eficiente na produção de espécies reativas de oxigênio (EROs). Para evitar os danos oxidativos causados pelas EROs, o Fe deve sempre estar ligado ou incorporado em estruturas (heme proteínas ou não-heme proteínas) que permitam o controle reversível das reações de redução e oxidação (Marschner, 1995).

O sistema empregado no cultivo do arroz no RS é principalmente por irrigação em solos alagadiços. Este ambiente caracteriza-se por apresentar baixas concentrações de oxigênio (anoxia) e baixo pH, o que facilita a redução do Fe^{+3} para Fe^{+2}, a forma mais facilmente absorvida pelas plantas. O mineral torna-se, assim, muito disponível

para absorção pela planta, aumentando excessivamente o conteúdo de Fe no vegetal. O Fe em excesso é extremamente tóxico, uma vez que pode agir como catalizador na formação de EROs. Através da reação de Fenton, o Fe^{+2} reage com H_2O_2 levando à oxidação do Fe (formação de Fe^{+3}) e geração do radical hidroxila, o agente oxidante mais potente que se conhece (Halliwell e Gutteridge, 1992; Becana et al. 1998; Briat, 2002). Esses radicais podem causar danos a diversas estruturas celulares como lipídios, proteínas e ácidos nucléicos, podendo levar à perda de integridade celular e morte (Guerinot e Yi, 1994; Briat et al. 1995). Os primeiros sintomas de toxidez por excesso de Fe são o bronzeamento das folhas (inicialmente as mais velhas), retardo no crescimento, baixa produtividade, esterilidade das espiguetas e, em casos mais severos, morte da planta (Ponnamperuma et al. 1955). Estima-se que o excesso de Fe pode levar a perdas de 15 a 30 % na lavoura de arroz, dependendo da região e da cultivar plantada (Lopes, 1987). Logo, tanto a manutenção dos níveis intracelulares de Fe quanto o armazenamento do Fe em formas não-tóxicas (ligado a quelantes orgânicos) devem ser processos finamente controlados (Morrissey e Guerinot, 2009).

Por outro lado, mesmo o Fe sendo o quarto elemento mais abundante na crosta terrestre, em solos com condições aeróbicas e pH neutro ou básico, a solubilidade torna-se muito baixa. Nessas condições, o Fe forma polímeros de óxido-hidróxidos altamente insolúveis. A concentração de Fe nessas condições é de aproximadamente 10^{-14} a 10^{-17} M, sendo que o ideal para que as plantas se desenvolvam adequadamente é entre 10^{-4} e 10^{-9} M (Guerinot e Yi, 1994). A deficiência de Fe é um grande problema para plantas que crescem em solos calcáreos, que representam 1/3 de todos os solos cultiváveis. A deficiência de Fe afeta diversos aspectos do desenvolvimento das plantas, incluindo a geração de zonas cloróticas intervenais e supressão do crescimento do meristema apical, levando à redução na produtividade e até mesmo perda da safra (Briat et al. 1995; Marschner, 2002; Curie e Briat, 2003; Larcher, 2003). Em condições extremas, pode levar a senescência e morte celular (Sperotto et al. 2007; Sperotto et al. 2008). A deficiência de ferro, além de ser um problema para as plantas, também é um enorme problema para a saúde humana, uma vez que os cereais são nossa principal fonte de ferro. A Organização Mundial da Saúde (WHO) considera esta a deficiência mineral de maior amplitude no planeta, sendo que mais de três bilhões de pessoas sofrem de deficiência de ferro (http://www.who.int/nutrition/publications/micronutrients/en/index.html). A principal conseqüência desta deficiência mineral é a anemia, que pode ser tratada com

suplementação alimentar. Porém, esta solução não é de fácil execução em países subdesenvolvidos, devido à falta de recursos financeiros e programas básicos de assistência médica. Por essas razões, é de extrema importância o entendimento dos mecanismos que controlam a absorção de ferro e a distribuição dentro dos vários órgãos das plantas cultivadas (Kerkeb e Connolly, 2006), visando o enriquecimento mineral com objetivos nutricionais.

Um dos principais sintomas da deficiência de ferro em plantas, a clorose (ou deficiência de clorofila) ocorre concomitantemente com decréscimo nas taxas fotossintéticas e mudanças na estrutura dos cloroplastos (Spiller e Terry, 1980), além de diminuição na expressão da Rubisco, de proteínas que se ligam à clorofila e enzimas envolvidas na síntese de clorofila (Spiller et al. 1987; Winder e Nishio, 1995; Belkhodja et al. 1998). A deficiência de ferro também altera a composição de lipídios e proteínas do tilacóide (Nishio et al. 1985); reduz a capacidade de transporte de elétrons no tilacóide (Spiller e Terry, 1980); e diminui os níveis de ATP nas folhas (Arulanantham et al. 1990), mas não afeta a atividade de enzimas chaves no processo de fotossíntese como gliceraldeído-3-fosfato desidrogenase e frutose-1,6-bifosfatase (Stocking, 1975; Taylor et al. 1982). 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).

Visto que tanto o excesso quanto a deficiência de ferro são prejudiciais ao crescimento vegetal, é de extrema importância desvendar os mecanismos ainda pouco conhecidos que regulam a homeostase deste metal.

Características gerais do Zinco

O zinco (Zn) é 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 e Helmke, 1993). Portanto, em contraste com o Fe, o Zn

não participa diretamente de reações redox (Marschner, 1995). O Zn tem uma forte tendência a 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 e 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 e 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 (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 e Richardson, 1980; Fraústo da Silva e Williams, 2001). 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+} (Irving e Williams, 1953); levando 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.

Absorção de ferro pelas plantas

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 desses metais: 18 genes YSL (*yellow stripe-like*), 2 FRO (*Fe^{+3} reductase*), 13 ZIP (*Zrt/ Irt-like protein*), 8 NRAMP (*natural resistance-associated macrophage protein*) e 2 FER (*ferritin*) (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. Esta lista inclui genes envolvidos com biossíntese de ácido muginéico (MA) e fitossideróforos (PS): 3 genes NAS (*nicotianamine synthase* - Inoue et al. 2003), 6 NAAT (*nicotianamine aminotransferase* - Inoue et al. 2008), DMAS (*deoximugineic acid synthase* - Bashir et al. 2006); genes responsáveis pela regulação gênica relativa à deficiência de Fe: IRO2 (*bHLH transcription factor* - Ogo et al. 2006), IDEF (*Fe-deficiency-responsive element factor* - Kobayashi et al. 2007); genes envolvidos com captura e translocação de metal: 13 ZIFL (*zinc-induced facilitator* - Haydon e Cobbett, 2007), MTP (*metal tolerance protein* - van der Zaal et al. 1999), 9 HMA (*heavy metal-associated domain* - Mills et al. 2003), FPN (*ferroportin* - Schaaf et al. 2006) e genes envolvidos com efluxo ou influxo de metais para as organelas: MIR (*mitochondrial iron-regulated gene* - Ishimaru et al. 2009), 2 PIC (*permease in chloroplasts* - Duy et al. 2007), 2 VIT (*vacuolar iron uptake transporter* - Kim et al. 2006).

O primeiro passo limitante para a acumulação de Fe nos grãos de arroz é a absorção deste nutriente pelas raízes. Ao contrário dos nutrientes orgânicos, os

nutrientes minerais (incluindo Fe e Zn) não podem ser sintetizados pelas plantas, sendo obtidos somente através da absorção do solo. O Fe (assim como Zn) é incapaz de se difundir através de membranas lipídicas, sendo necessária a ação de proteínas transportadoras para que esses metais entrem na planta. Embora o Fe seja abundante no solo, sua disponibilidade para as plantas normalmente é muito baixa, principalmente pela insolubilidade do Fe^{+3} (Lemanceau et al. 2009).

Em condições de suficiência de Fe, as plantas reduzem Fe^{+3} e transportam o Fe^{+2} resultante através da membrana plasmática via um transportador de baixa afinidade, ainda não caracterizado em nível molecular (Curie e Briat, 2003). Em condições de deficiência de Fe, as plantas desenvolveram diferentes estratégias para aumentar a absorção deste nutriente, as chamadas estratégias I e II (Marschner e Römheld, 1994).

A estratégia I é utilizada por dicotiledôneas e monocotiledôneas não-gramíneas e consiste em três processos coordenadamente induzidos nas raízes (Palmer e Guerinot, 2009): (1) extrusão de prótons por bombas dependentes de ATP para acidificar a rizosfera e aumentar a solubilidade do Fe^{+3} (codificada pelo gene *AtAHA2*, *A*rabidopsis H^+-pump ATPase; Santi et al. 2009); (2) redução do Fe^{+3} por redutases férricas ligadas à membrana (codificada pelo gene *AtFRO2*, Ferric Reductase Oxidase; Robinson et al. 1999); e (3) absorção do Fe^{+2} por transportadores transmembrana de Fe (codificado pelo gene *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, 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 fitossideróforos (PS), os quais são derivados estruturais do ácido muginéico e apresentam uma alta afinidade por Fe^{+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: S-adenosil-metionina sintetase (SAMS; Takizawa et al. 1996); nicotianamina sintase (NAS; Higuchi et al. 1999); nicotianamina aminotransferase (NAAT; Takahashi et al.

1999); ácido deoximuginéico 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 em 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 da proteína Yellow Stripe 1 de milho (YS1; Curie et al. 2001).

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 1; Walker e 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 (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 e OsIRT2, 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 e Guerinot, 2009).

Absorção 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 e 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 e 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 dessas ou de outra família foi caracterizado como tendo essa função até o momento (Palmgren et al. 2008).

Transporte de íons metálicos via xilema

Depois de absorvido do solo por transportadores localizados nas membranas das células da raiz, os metais são transportados via xilema até os órgãos vegetativos através da via transpiratória, onde podem ser temporariamente estocados no colmo ou tecidos foliares e posteriormente mobilizados para as sementes via floema (Grusak e DellaPenna, 1999). Diversas evidências apontam o citrato como sendo o principal quelante de Fe no xilema (Cataldo et al. 1988; Abadía et al. 2002). Foi demonstrado que AtFRD3 (Ferric Reductase Defective), uma proteína que pretence à família MATE (Multidrug and Toxin Efflux), é capaz de transportar um quelante de Fe no xilema das raízes, promovendo a saída de Fe dos vasos do xilema e a entrada nas células foliares de *Arabidopsis* (Rogers e Guerinot, 2002; Green e Rogers, 2004). Também foi demonstrado que AtFRD3 é capaz de mediar o efluxo de citrato para os tecidos vasculares da raiz, um processo importante na translocação de Fe para as folhas. Estes resultados confirmam estudos anteriores que sugeriam que o Fe move-se através do xilema como Fe^{+3} complexado com citrato (Durrett et al. 2007). Recentemente, Yokosho et al. (2009) mostraram que o ortólogo de AtFRD3 em arroz, OsFRDL1 (*FRD-like*), também é um transportador de citrato localizado nas células do periciclo, e é necessário para a eficiente translocação de Fe para as partes aéreas. 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).

OsFRDL1 poderia ser um importante alvo para manipulação do conteúdo de Fe em sementes de arroz, uma vez que, ao contrário do trigo (*Triticum* spp.) (Zee e O'Brien, 1970), o arroz não possui a descontinuidade do xilema na base de cada grão (Zee, 1972; Krishnan e Dayanandan, 2003). Dessa forma, o Fe (e também o Zn) poderia ser transportado diretamente do xilema para o grão (Stomph et al. 2009). Entretanto, ainda não se sabe se o movimento de Fe e Zn através da raiz e do xilema pode ser um passo limitante na acumulação desses metais nos grãos de arroz. Mutantes de ervilha

que exibem elevada capacidade de absorção de Fe apresentam hiperacumulação de Fe apenas nas folhas e não nas sementes (Kneen et al. 1990). Esse passo limitante pode ser diferente entre monocotiledôneas e dicotiledôneas, e mesmo entre gramíneas, e essas diferenças são provavelmente causadas por diferenças nas estruturas anatômicas (Thorne, 1985).

Transporte de íons metálicos via floema

A quantidade de metais remobilizados via floema é fortemente dependente da mobilidade de cada elemento no floema. O Fe é descrito como tendo mobilidade intermediária em plantas, enquanto que o Zn é considerado altamente móvel (Marschner, 1995). Utilizando os mutantes de ervilha *brz* (*bronze*) e *dgl* (*degenerated leaflets*) hiperacumuladores de Fe, foi demonstrado que o Fe deve ser quelado antes de ser carregado via floema, visto que íons metálicos precipitam em pH alcalino, como encontrado no floema (Grusak, 1994). Este fato aponta para a necessidade de quelação para garantir o transporte desses metais através do floema (Zhang et al. 2007).

Entre os quelantes conhecidos, a nicotianamina (NA), um aminoácido não proteinogênico, é um dos mais estudados. A NA forma complexos estáveis com o Fe e outros íons metálicos bivalentes (Benes et al. 1983; Anderegg e Ripperger, 1989; Curie et al. 2009) e tem um papel crucial no transporte interno de Fe e outros metais como Cu e Zn (Stephan et al. 1994; Pich e Scholz, 1996; Takahashi et al. 2003). Por formar complexos altamente estáveis em pH alcalino, é um forte candidato para atuar como quelante de metais no transporte de longa distância através do floema (von Wirén et al. 1999; Koike et al. 2004; Curie et al. 2009). Além do transporte realizado através da complexação com NA, o Fe é descrito como sendo transportado através do floema por polipeptídeos e proteínas. Em *Ricinus communis* foi identificada uma proteína de 2,4 kDa que liga-se especificamente a Fe^{+3} e não a Fe^{+2}, denominada ITP (*iron transport protein*), que é capaz de ligar-se também a outros metais como Cu^{+2}, Zn^{+2} e Mn^{+2} (Kruger et al. 2002).

A família de transportadores YSL (*yellow stripe-like*) contém sérios candidatos para o transporte de metais complexados com NA através do floema. Evidências experimentais apontam as proteínas YSL funcionando como transportadores intracelulares e de longa distância de metais complexados com NA, principalmente Fe

(Curie et al. 2009). Em *Arabidopsis*, dois mutantes perda de função *ysl1* exibiram aumento no acúmulo de NA nas partes aéreas, e as sementes dos mutantes continham menos Fe e NA do que as sementes selvagens, mesmo em condições de excesso de Fe (Le Jean et al. 2005). Esse padrão é consistente com o co-transporte de Fe e NA, e esse transporte parece depender da proteína AtYSL1. Em sementes de plantas do duplo mutante *ysl1ysl3*, as concentrações de Fe, Zn e Cu são menores quando comparadas com as sementes de plantas selvagens e a mobilização de metais a partir das folhas durante a senescência é diminuída no duplo mutante (Waters et al. 2006).

Em arroz, a proteína OsYSL2 foi sugerida como responsável pelo transporte de Fe(II)-NA através do floema, participando dessa forma na translocação de Fe para o grão (Koike et al. 2004). Um dos três genes de arroz que codificam NA sintase (*OsNAS3*) é expresso somente no floema (Inoue et al. 2003), fornecendo a NA necessária pra quelar os metais de transição antes de sua entrada no feixe vascular do floema. Estes resultados claramente estabelecem um papel para a NA no transporte de metais através do floema e das proteínas YSL no transporte de longa distância de metais complexados a NA, incluindo o transporte até os grãos.

Remobilização de íons metálicos das folhas para os grãos

Os processos responsáveis pela disponibilização e remobilização de nitrogênio para os grãos têm sido bastante estudados (Hortensteiner e Feller 2002; Schiltz et al. 2005). Durante o enchimento do grão, os assimilados são preferencialmente alocados para as sementes. A demanda de nitrogênio das sementes é parcialmente suprida pela absorção através das raízes, mas parte do nitrogênio é remobilizado a partir dos órgãos vegetativos. Nas folhas, grande parte do nitrogênio se origina da maquinaria fotossintética e da degradação de proteínas do cloroplasto que ocorre durante a senescência foliar (Burstin et al. 2007). Dessa forma, a regulação do início da senescência é importante para determinar o conteúdo de nitrogênio no grão. Em trigo, o acúmulo de nitrogênio nos grãos é dependente de eventos que ocorrem nos tecidos dreno (grãos em desenvolvimento) e fonte (folhas). Entretanto, Martre et al. (2003) sugeriram que os eventos que ocorrem nos tecidos fonte têm papel principal no acúmulo de proteínas nos grãos.

Além do nitrogênio, minerais também podem ser remobilizados de tecidos vegetativos (Hocking e Pate, 1977; Himelblau e Amasino, 2001), embora grande parte dos minerais presentes nas sementes sejam originados da contínua absorção e translocação durante o crescimento reprodutivo. Em trigo, remobilização de Zn (Hocking, 1994; Ozturk et al. 2006) e Fe (Garnett e Graham, 2005) a partir das folhas foi observada. De acordo com Jiang et al. (2007b), em plantas de arroz crescendo em suficiência ou excesso de Zn, a maior parte do Zn acumulado nos grãos origina-se da absorção pelas raízes após o florescimento, e não da remobilização de Zn das folhas. Entretanto, receentemente Wu et al. (2010) mostraram que mais da metade do Zn presente nos grãos de arroz foi absorvido no estágio vegetativo, mostrando que grandes quantidades de Zn foram remobilizadas a partir de outras partes da planta e não transportadas para os grãos diretamente após a absorção pelas raízes. Recentemente, Waters e Grusak (2008) sugeriram que, em *Arabidopsis*, a contínua absorção e translocação de minerais durante o enchimento do grão é tão importante, ou mais importante, que a remobilização de minerais estocados. A quantidade de minerais remobilizados via floema depende da mobilidade de cada elemento. O Fe, apesar de apresentar mobilidade intermediária (Marschner, 1995) em condições normais, pode ter sua remobilização das folhas para as sementes aumentada durante processos de senescência (Zhang et al. 1995). Por exemplo, uma grande remobilização de Fe das partes aéreas para os grãos foi vista em trigo, sendo que 65 a 77% do Fe total presente nas partes aéreas foi encontrado nos grãos maduros (Garnett e Graham, 2005; Waters et al. 2009).

As folhas-bandeira (que se encontram mais próximas da panícula) são as principais fontes de fotoassimilados para as sementes em desenvolvimento, e acredita-se que contribuam na remobilização de metais para as sementes (Grusak e DellaPenna, 1999; Narayanan et al. 2007). Não existem na literatura trabalhos que mostrem translocação de Fe e Zn das folhas-bandeira para as sementes de arroz. Assim, a importância relativa da remobilização de metais da folha-bandeira em comparação com outras possíveis fontes de metais para o grão (como a remobilização de outras folhas, o transporte diretamente via xilema ou via floema) ainda não está estabelecida para plantas de arroz. Entretanto, remobilização de Fe e Zn das folhas-bandeira para as sementes já foi mostrada em trigo (Uauy et al. 2006; Waters et al. 2009). De acordo com Jiang et al. (2007b), uma pequena proporção do Zn marcado radioativamente fornecido às folhas-bandeira foi encontrado nos grãos de arroz e, em condições de

deficiência de Zn, o conteúdo desse metal diminui nas folhas-bandeira durante o enchimento do grão do arroz, sugerindo que pelo menos uma pequena parte do Zn presente nos grãos tem sua origem nas folhas-bandeira (Jiang et al. 2008).

É possível que outros mecanismos de alocação de metais para os grãos de arroz, como remobilização a partir de outros tecidos, o transporte diretamente via xilema ou via floema, possam contribuir com maiores quantidades de Fe e Zn do que a remobilização a partir das folhas-bandeira durante o enchimento do grão de arroz. Entretanto, um aumento significativo na remobilização das folhas-bandeira pode ter um impacto positivo nos esforços de biofortificação e pode ser alcançado no futuro, desde que os genes importantes para este processo sejam identificados e estudados. A caracterização desses genes-alvo é de extrema importância para utilização em programas de melhoramento tradicional ou transgenia. Esses alvos devem incluir não apenas genes que aumentem a absorção de Fe e Zn, mas também genes que codifiquem transportadores internos, como os responsáveis pelo carregamento através dos tecidos vasculares e pelo influxo ou efluxo das organelas, além de fatores de transcrição que regulem esses processos.

Objetivos

Apesar de todos os avanços recentes na compreensão da homeostase de metais e remobilização destes para os grãos de arroz, muitas questões ainda precisam ser 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 e Guerinot, 2009). Igualmente, ainda não estão totalmente elucidados os sistemas de transporte de metais para organelas, nas quais o Fe e o Zn exercem funções essenciais (Raven et al. 1999; Lister et al. 2002; Moberg et al. 2003). Dessa forma, o objetivo geral deste trabalho é identificar genes importantes para a translocação de Fe e/ou Zn para os grãos de arroz.

Os objetivos específicos desse trabalho são:

- identificar cultivares de arroz com níveis contrastantes de Fe e/ou Zn nos grãos;

- identificar genes com expressão aumentada em folhas-bandeira na fase de enchimento de grão (R5), em relação à fase de emergência da panícula (R3), através da técnica de SSH (*suppression subtractive hybridization*);

- confirmar os diferentes níveis de expressão dos genes encontrados por SSH usando PCR quantitativo em cultivares com níveis contrastantes de Fe e/ou Zn no grão;

- quantificar a expressão de diversos genes relacionados à homeostase de Fe e/ou Zn em folhas-bandeira de oito cultivares de arroz (com níveis contrastantes de Fe e/ou Zn nos grãos), em duas fases de desenvolvimento: R3 (emergência da panícula) e R5 (enchimento do grão);

- correlacionar os níveis de expressão desses genes em folhas-bandeira com as concentrações finais de Fe e Zn nos grãos.

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ORIGINAL ARTICLE

Identification of up-regulated genes in flag leaves during rice grain filling and characterization of *Os*NAC5, a new ABA-dependent transcription factor

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Abstract Rice is a poor source of micronutrients such as iron and zinc. To help clarify the molecular mechanisms that regulate metal mobilization from leaves to developing seeds, we conducted suppression subtractive hybridization analysis in flag leaves of two rice cultivars. Flag leaves are the major source of remobilized metals for developing seeds. We isolated 78 sequences up-regulated in flag leaves at the grain filling stage relative to the panicle exertion stage. Differential expression of selected genes (encoding 7 transport proteins, the *Os*NAS3 enzyme and the *Os*NAC5 transcription factor) was confirmed by quantitative RT-PCR. We show that *Os*NAC5 expression is up-regulated by natural (aging) and induced senescence processes (dark, ABA application, high salinity, cold and Fe-deficiency) and its expression is not affected in the presence of 6-benzylaminopurine (a senescence inhibitor) under dark-induced senescence. Salt induction of *Os*NAC5 expression is abolished by nicotinamide, an inhibitor of ABA effects. This result and the presence of *cis*-acting elements in the promoter region of the *Os*NAC5 gene suggest an ABA-dependent regulation. Using four different rice cultivars, we show that *Os*NAC5 up-regulation is higher and earlier in flag leaves and panicles of IR75862 plants, which have higher seed concentrations of Fe, Zn and protein. We suggest that *Os*NAC5 is a novel senescence-associated ABA-dependent NAC transcription factor and its function could be related to Fe, Zn and amino acids remobilization from green tissues to seeds.

Keywords ABA-responsive · Correlation analyses · Flag leaves · Grain filling · Metal mobilization · Senescence · Suppression subtractive hybridization

Abbreviations

ABA	Abscisic acid
ABC	ATP-binding cassette
BAP	6-Benzylaminopurine
GST1	Glutathione-*S*-transferase
ICP-OES	Inductively coupled plasma optical emission spectroscopy
MES	2,4-Morpholino-ethane sulfonic acid
MST	Monosaccharide transporter
NA	Nicotianamine
NAS	Nicotianamine synthase
OPT	Oligopeptide transporter
PTR	Peptide transporter
SGR	Staygreen
SSH	Suppression subtractive hybridization
STP	Sugar transport protein

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Introduction

Rice is the staple food of half of the world's population. To keep up with the population growth and to improve the nutrition and health of rice consumers, development of high-quality rice varieties becomes increasingly important (Duan and Sun 2005). Micronutrient malnutrition, and particularly Fe and Zn deficiencies, affect over 3 billion people worldwide, mostly in developing countries (Welch and Graham 2004). According to the World Health Organization, it is estimated that more than 160 million children under the age of five lack adequate levels of protein (WHO—Meeting of Interested Parties: Nutrition, 2001, http://www.who.int/mip2001/files/2299/MIP_01_APR_SDE_3.en.pdf). Biofortification has emerged as one possible solution to alleviate malnutrition. Biofortification consists of the use of plant breeding and/or transgenic approaches to develop new cultivars with the potential to increase the nutrient concentration of edible portions of crop plants (White and Broadley 2005). In the recent years, several studies have provided information about the physiology and regulation of mineral uptake from the rhizosphere. However, the lack of knowledge about how nutrients are moved into or out of vascular tissues, translocated to vegetative tissues and loaded into seeds is one of the barriers to biofortification of seeds (Colangelo and Guerinot 2006; Kramer et al. 2007). Initial seed biofortification efforts for Fe and Zn in rice have focused on increasing the iron storage protein ferritin (Goto et al. 1999; Vasconcelos et al. 2003) or root ferric reductase activity (Vasconcelos et al. 2004). The transgenic plants showed an overaccumulation of minerals in leaves, but only a small increase in seeds. Analysis of mineral overaccumulation mutants indicates that translocation of minerals to seeds is tightly regulated, and that simply increasing uptake into the plant will probably not result in seeds with higher mineral concentrations. These results suggest that additional regulatory mechanisms must be manipulated to improve remobilization and pass through of minerals from leaves into seeds.

The source tissues and processes responsible for the remobilization and supply of nitrogen to seeds has received considerable attention (Hortensteiner and Feller 2002; Schiltz et al. 2005). During grain filling, seeds are the main sink to which assimilates are preferentially allocated at the expense of vegetative organs. Seed nitrogen demand is partly satisfied by nitrogen acquired by the roots, but also by nitrogen remobilized from almost all vegetative organs. In leaves, most of the translocated N originates mainly from degradation of chloroplast proteins and photosynthetic machinery occurring during leaf senescence (Burstin et al. 2007). Optimal regulation of the onset and rate of senescence is therefore important to determine grain N content. In wheat, the accumulation of nitrogen in the grain is likely to be the result of events happening both at the sink (developing grains) and at the source (leaves). However, Martre et al. (2003) suggest that source regulation plays a major role in grain protein accumulation. Minerals other than nitrogen may be remobilized from vegetative sources (Hocking and Pate 1977; Himelblau and Amasino 2001), although a major portion of minerals in seeds are likely supplied through continuous uptake and translocation during reproductive growth to developing seeds. In wheat, Zn (Hocking 1994) and Fe (Garnett and Graham 2005) remobilization from leaves was observed. According to Jiang et al. (2007b), in rice plants grown under sufficient or surplus Zn supply, most of the Zn accumulated in the grain originates from uptake by roots after flowering and not from Zn remobilization from leaves. Recently, Waters and Grusak (2008) suggested that, in *Arabidopsis*, continuous uptake and translocation of minerals to source tissues during seed fill are as important, if not more important, than remobilization of previously stored minerals. The amount of minerals remobilized via the phloem is greatly dependent on the phloem mobility of each element. Zinc has been described as having good remobilization from wheat leaves (Pearson and Rengel 1994) and iron has been described as intermediately mobile in plants (Marschner 1995). However, it is already known that Fe remobilization from leaves to seeds can be increased during senescence (Zhang et al. 1995).

The transition from vegetative development to reproductive growth is a complex process involving physiological, biochemical, and gene expression changes that are regulated by endogenous and exogenous factors (Lim et al. 2007). The onset of senescence represents the end of the functional life of leaves and the entrance into a catabolic phase that ends with cell death and degradation. This process involves highly regulated and orderly molecular and cellular events that allow the plant to mobilize nutrients and metabolites from source to sink tissues. Therefore, deciphering the molecular control of the onset of senescence and the variable range of transporters, which are responsible for the movement of nutrients from source to sink tissues will be important for genetic improvement of rice grain nutrient composition.

Rice flag leaves are the major source of phloem-delivered photoassimilates for developing seeds, and are also believed to be a major source of remobilized metals to seeds (Grusak and DellaPenna 1999; Narayanan et al. 2007). Since we were interested in understanding how nutrients are mobilized from flag leaves to seeds, we compared gene expression in flag leaves of two rice cultivars containing high grain iron concentration. We constructed a flag leaf SSH library using mRNA extracted from R3 (panicle exertion) flag leaves as driver and from R5 (grain filling) flag leaves as tester. Here, we report the identification

of several genes up-regulated during rice grain filling, including *Os*NAC5. We provide evidence that *Os*NAC5 is a senescence-associated gene (SAG) regulated by abscisic acid (ABA). We suggest that *Os*NAC5 could be involved in the senescence process and in nutrient remobilization from flag leaves to developing grains.

Materials and methods

Plant growth conditions

Plants from eight rice (*Oryza sativa* L.) cultivars [IR69428 and IR75862 (2 *japonica* cultivars) and Canastra, Epagri 108, BR-IRGA421, BR-IRGA409, IR68144 and IR68144_1 (6 *indica* cultivars)] were grown in soil under flooded conditions in an experimental unit of Instituto Rio Grandense do Arroz (IRGA), in Cachoeirinha, RS, Brazil (29°54′ 58.61″S 51°10′ 02.65″W), during the rice growing season (October 2007 to March 2008). Soil characteristics of this site were reported by Stein et al. (2009). Tissues were collected during R3 (panicle exertion), R4 (anthesis), R5 (grain filling), R7 (grain dry down) and R9 (full maturity) stages (Counce et al. 2000), and immediately frozen in liquid nitrogen. For each organ type, three samples were collected from a plant with multiple tillers and samples were pooled. Three biological replicates were collected for use with the quantitative PCR studies. Seeds were also harvested from plants grown to full maturity for mineral analysis.

In laboratory experiments, rice seeds from IR75862 cultivar were germinated for 4 days in an incubator (28°C, first 2 days in the dark and last 2 days in the light) on filter paper soaked with distilled water. After germination, plants were transferred to holders positioned over glass pots covered with aluminum foil and containing one liter of nutrient solution (as described by Ogo et al. 2006). All solutions were replaced every 3 days. Plants were cultivated in a growth room at 26 ± 1°C under white light with a photoperiod of 16/8 h light/dark (irradiance of approximately 100 μmol m^{-2} s^{-1}). For treatments involving Fe-deficiency, growth conditions were exactly the same as those used by Ogo et al. (2006). Fe-deficiency was initiated 3 weeks after germination by omitting Fe(III)-EDTA from the growth medium. Plants from cultivar IR75862 were harvested after 1, 4, 7 and 21 days of Fe-deficiency treatment.

Elemental analysis by ICP

Whole seeds and flag leaves from nine plants were harvested and dried in a 60°C oven for 48 h. Dried tissues were predigested overnight in borosilicate glass tubes with 4 ml of redistilled 98.8% HNO_3. 1 ml of concentrated trace metal grade $HClO_4$ was added to the predigested tissues and heated at 100°C for 1 h, 150°C for 1 h, 180°C for 1 h and then at 210°C to dryness (1–2 h). Digestions were performed using a heating block (Model 1016, Tecator, Hoganas, Sweden) with an exhaust-collecting manifold. Digests were resuspended in 15 ml of redistilled 2% HNO_3. Elemental analysis was performed using inductively coupled plasma-optical emission spectroscopy (CIROS ICP Model FCE12; Spectro, Kleve, Germany). Tomato leaves and rice flour standards (SRM 1573A and 1568A, respectively; National Institute of Standards and Technology, Gaithersburg, MD, USA) were digested and analyzed along with the rice samples to ensure the accuracy of the instrument calibration (Narayanan et al. 2007).

Soluble protein concentration

Soluble proteins were extracted from rice seeds according to Xu et al. (2008). Rice seeds were homogenized in 20 mM sodium acetate buffer, pH 5.0, containing 250 mM NaCl, followed by centrifugation at 13,000 rpm for 20 min at 4°C. The supernatant was used for the determination of protein concentrations using the Quant-iT Protein Assay Kit and the Qubit fluorometer (Invitrogen, Carlsbad, CA, USA).

RNA extraction and cDNA synthesis

Rice tissues were harvested from plants grown under field or laboratory conditions. Total RNA was extracted using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Mountain View, CA, USA), according to the manufacturer's instructions, in the presence of RNase OUT (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA, USA) using 1 μg of RNA.

Construction of subtractive cDNA libraries

Suppression subtractive hybridization (SSH) was carried out as described by Diatchenko et al. (1996) using the PCR-Select cDNA Subtraction Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. First-strand cDNA (2 μl) was used as template to synthesize the second strand of cDNA. Tester (R5, grain filling stage) and driver (R3, panicle exertion stage) cDNAs were digested with *Rsa* I and ligated to adaptors. Two rounds of hybridization and PCR amplification were performed to enrich the differentially expressed sequences. 100 ng of the

subtracted cDNAs was purified and cloned into the pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligated products. Individual bacterial colonies were picked and grown in 96-well plates. Plasmid DNA preparation and sequencing of 576 selected clones were performed by *ht*SEQ (High-Throughput Sequencing Solutions, Department of Genome Sciences, University of Washington, USA, http://www.htseq.org/). The vector sequence was removed manually and the resulting sequences were matched to rice full-length cDNA sequences available at the Rice Pipeline (http://cdna.01.dna.affrc.go.jp/PIPE/), a unification tool that dynamically integrates data from various databases (Yazaki et al. 2004).

Quantitative RT-PCR and data analysis

Quantitative RT-PCRs were carried out in an Applied-Biosystem 7500 real-time cycler. All primers (listed in Table 1) were designed to amplify 100–150 bp of the 3′-UTR of the genes and to have similar T_m values (60 ± 2°C). Reaction settings were composed of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C, 15 s at 72°C and 35 s at 60°C (fluorescence data collection); samples were held for 2 min at 40°C for annealing of the amplified products and then heated from 55 to 99°C with a ramp of 0.1°C/s to produce the denaturing curve of the amplified products. qRT-PCRs were carried out in 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 100 times, 2 µl of 10× PCR buffer, 1.2 µl of 50 mM $MgCl_2$, 0.1 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 4.25 µl of water, 2.0 µl of SYBR green (1:10,000, Molecular Probe), and 0.05 µl of Platinum Taq DNA polymerase (5 U/µl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated by the $2^{-\Delta CT}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). For each sample, analyzed in four technical replications, a ΔC_T value was obtained by subtracting the Ubiquitin C_T value from the C_T of the gene of interest. Each data point corresponds to three true biological replicate samples.

Dark and hormonal regulation of leaf senescence

All analyses were performed with fully expanded leaves of 2-week-old plants grown in hydroponic condition, according to Kusaba et al. (2007). Detached leaves (approximately 0.5 cm^2) were incubated in 3 mM MES (2,4-morpholino-ethane sulfonic acid) buffer, pH 5.8, at 27°C in darkness. A concentration of 50 µM abscisic acid (ABA, inductor of senescence) or 50 µM 6-benzylaminopurine (BAP, inhibitor of senescence) was added to the MES solution in the phytohormone treatments. Control leaves were maintained in MES solution. Samples were collected after 0, 1, 2, and 3 days of treatment.

Stress treatments

Two-week-old IR75862 rice plants grown in hydroponic condition were submitted to high salinity (final concentration of 100 mM NaCl), cold (4°C), high temperature (40°C) or high salinity + 50 mM nicotinamide (Fluka Chemie AG, Buchs, Switzerland) for 4 days. Control plants were maintained in nutrient solution (Ogo et al. 2006). Control and treated plants were cultivated in growth chambers under white light with a photoperiod of 16/8 h light/dark (irradiance of approximately 100 µmol m^{-2} s^{-1}). Fully expanded leaves were collected after 0, 1 and 4 days of the onset of

Table 1 Gene-specific PCR primers used for qRT-PCR

Gene	Forward primer 5′ → 3′	Reverse primer 5′ → 3′
*Os*NAS3	AGGAGGAGGAGGTGATCGAGA	CTGCTCCACGTCCACGCT
*Os*NAC5	CAGCAGCTGATGGTATTGTC	AGAGACCTGTTTGGCACGAA
*Os*GST1	AACGATTCGTCCGTTGTTTC	GAATCGGAGGCAACTCTCTG
*Os*PTR	GACCCACCCAAAAATGTCAC	TGCTTTGTGTCTGAGGTTGC
*Os*STP	CGAATTTCGTAATGTCGAGG	GTTTGTACGTCGCTGGCATA
*Os*MST1	ATGTCTGCCCGTTTTACTGC	GCAGAAACTGCTTGGGAGAA
*Os*MST3	CTTGTGTTCATCCCCGTGTA	TTGGCCAAACAAGAAACACA
ABC (J013072F03)	GGTGTGTGTAGGCGTATATTGG	CAAAGGCTTCTGGTCAAAGG
ABC (J023069G03)	TGGACGTTTTCCTAGCCTTG	TTTCTAGTGTGGCTACGCATTC
ABC (J023056A15)	CCCCCAAATAGTACCACTGC	CATGATTCCCGAAAAGAACTG
*Os*SGR	CTACCAAACCGAGCCAAAAT	ACCAAAACGACTCTTGACAGC
*Os*IRO2	CCACAGGAAGCTCAGCCACA	CAGATTCTCCACCTGCTTCTGC
ONAC010	CATATGGCTTTGCCTTCACA	ATGGTACAAACGCATGAAGC
*Os*UBQ	AACCAGCTGAGGCCCAAGA	ACGATTGATTTAACCAGTCCATGA

the treatments. Treatments were initiated 8 h into the photoperiod.

*Os*NAC5 gene promoter analysis

A 1,500 nucleotide upstream region of the transcription initiation site of the *Os*NAC5 gene (NCBI code AK064292) was analyzed to search for *cis*-regulatory elements in the PLACE database (Higo et al. 1999), using the Group Signal Scan tool. The nucleotide positions were determined according to the sequence deposited at the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) as LOC_Os11g08210.

NAC domain analysis

Alignments of the NAC domain of proteins ONAC010 (NP_911241), *Tt*NAM-B1 (DQ869673) and *Os*NAC5 (AB028184) was performed with CLUSTALW (http://www.ebi.ac.uk/clustalw/) and BOXSHADE was used to produce the graphical representation (http://www.ch.embnet.org/software/BOX_form.html). The secondary structure was obtained from an analysis with PDB Sum (http://www.ebi.ac.uk/pdbsum/) and ProMotif tool.

Statistical analyses

When appropriate, data were subjected to analyses of variance (ANOVA) and mean values were compared by the Tukey Honestly Significant Differences (HSD) or Student's *t* test ($P \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., USA). The Levene's test (for homogeneity of variance) was used prior to ANOVA. All the analyses resulted in confirmation of homogeneity, except for *Os*SGR expression levels in Fig. 9a, which were then transformed in square root values. Pearson's correlation analyses were carried out using two significance levels ($P \leq 0.05$ and 0.01).

Results and discussion

Fe, Zn and protein concentrations in seeds

Elemental analysis was performed on mature seeds (R9 stage) of each cultivar by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Fig. 1a, b). Canastra and Epagri 108 plants were found to have a significantly lower seed iron concentration ($P \leq 0.05$) relative to the other cultivars (Fig. 1a). Seed zinc concentration was higher in IR68144_1, IR69428 and IR75862 than in the other five cultivars. The lowest Zn concentration was found in Epagri 108 and intermediate levels in

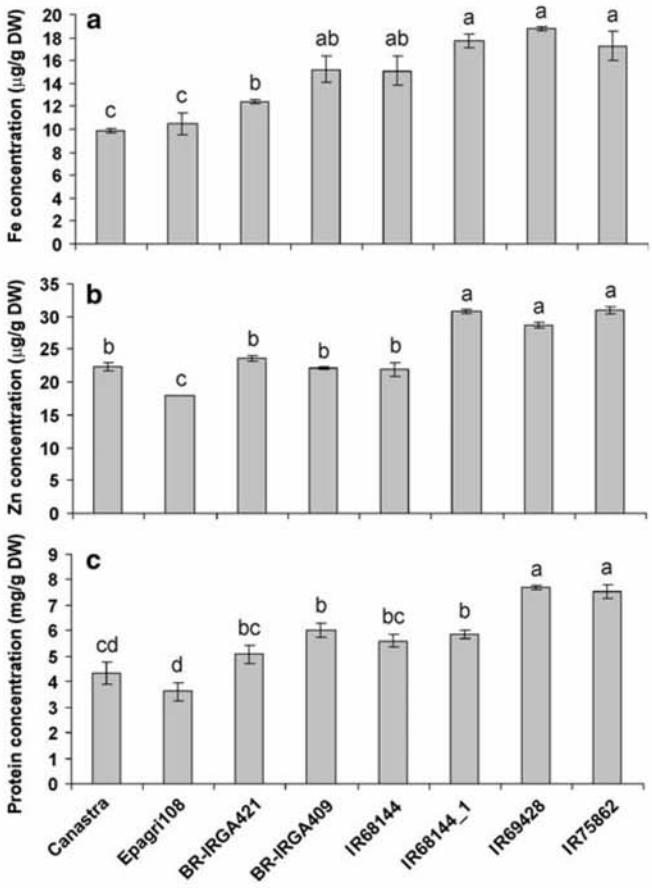

Fig. 1 Iron (**a**), Zinc (**b**) and protein (**c**) concentrations in whole seeds from eight diverse rice cultivars. Seeds were collected at full maturity (R9 stage). Values are the averages of three samples ± SE. Mean values indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). *DW* dry weight

Canastra, BR-IRGA421, BR-IRGA409 and IR68144 (Fig. 1b). Soluble protein concentration was also quantified on mature seeds collected from each of the cultivars. Seed protein concentrations were significantly higher in IR69428 and IR75862 ($P \leq 0.05$) relative to the other cultivars (Fig. 1c). In order to find relationships between minerals and protein concentration in seeds, Pearson's correlation analyses were performed. There were positive correlations between Fe, Zn and protein concentrations (Table 2). No correlations were found for other metals (Cu, Mn and Mo) with protein concentration. Positive correlation between Zn and Fe concentrations was previously reported in wheat (Cakmak et al. 2004; Morgounov et al. 2007) and rice (Jiang et al. 2007a). Mg, Zn, Cu, and Mn contents were positively correlated with protein content in rice seeds (Jiang et al. 2007a) and Distelfeld et al. (2007) showed a clear association between protein, Fe, Zn and Mn concentrations in wheat grains. To the best of our knowledge, this is the first report of positive correlation between protein, Fe and Zn concentrations in rice seeds,

Table 2 Correlation analyses between protein, Fe, Zn, Cu, Mn and Mo concentrations in seeds from eight rice cultivars (Canastra, Epagri 108, BR-IRGA421, BR-IRGA409, IR68144, IR68144_1, IR69428 and IR75862)

	Protein	Fe	Zn	Cu	Mn
Fe	0.908**				
Zn	0.799**	0.806**			
Cu	0.388	0.499	0.274		
Mn	−0.195	−0.337	−0.121	−0.858**	
Mo	−0.204	−0.446	−0.214	−0.750**	0.930**

Cu, Mn and Mo concentrations are shown in Supplementary Fig. 1

** Significant at 0.01 probability level

suggesting that higher protein, Fe and Zn concentrations may occur simultaneously in rice.

Since the highest Fe, Zn and protein concentrations were seen in the *japonica* IR75862 cultivar, we decided to perform SSH analysis with flag leaves of this cultivar to search for genes with higher expression during the grain filling stage (R5) relative to the panicle exertion stage (R3). SSH analysis was also performed with the *indica* IR68144 cultivar due to its high Fe and intermediate Zn and protein concentrations in seeds.

Construction of the SSH library

The SSH method was chosen over other existing methods such as differential display because it is a powerful tool to identify abundant differentially expressed genes and also to enrich the library for genes with low expression level (Diatchenko et al. 1996). This method is based on the generation of libraries of differentially expressed clones by subtraction of tester cDNA (in our case, flag leaves in R5 stage) with an excess of driver cDNA (flag leaves in R3 stage). Subtracting R3 from R5 mRNA populations provided 78 unique cDNAs. Among these, 61 cDNAs (78%) have putative functions and 17 cDNAs (22%) could not be assigned any function (Supplementary Table 1). Of the 78 sequenced clones, 27 were identified only in the *indica* IR68144 rice cultivar and 31 only in the *japonica* IR75862 rice cultivar. Twenty sequences were identified in both cultivars. The 78 sequences obtained were grouped into 10 functional categories, including transport, transcription factors, photosynthesis/photorespiration, protein biosynthesis, protein modification, protein degradation, structural, lipid metabolism, others and unknown (Supplementary Table 1).

Up-regulated genes in rice flag leaves during grain filling

Using the SSH method, we detected transcripts for 11 potential transport proteins, including 2 monosaccharide transporters (*Os*MST1 and 3), glutathione-*S*-transferase (*Os*GST1), sugar transport protein (*Os*STP), peptide transporter (*Os*PTR) and 3 ABC-type transport proteins (Table 3 and Supplementary Table 1).

Plant MSTs mediate transport of a variable range of monosaccharides across membrane barriers. Although most of the isolated monosaccharide transporters are preferentially expressed in sink organs, some MSTs that exhibit expression both in sink and source organs have been found in tomato (Gear et al. 2000), grapevine (Hayes et al. 2007) and rice (Wang et al. 2007). According to Toyofuku et al. (2000), the amino acid residues considered as the motifs of the sugar transport are highly conserved in *Os*MST1 and *Os*MST3 sequences. However, while *Os*MST1 did not show any glucose transport activity in yeast cells, *Os*MST3 activity was nearly three times higher than in cells transformed with the empty vector. This monosaccharide

Table 3 Up-regulated genes in rice flag leaves during the grain filling (R5) stage

Functional categories	Rice pipeline clone number	Description	IR68144 (*indica*)	IR75862 (*japonica*)	E-value
Transport	J013003K16	Monosaccharide transporter 1 (OsMST1)	X	–	0.0
	J033117G08	Monosaccharide transporter 3 (OsMST3)	–	X	1e-108
	001-123-F01	Glutathione-S-transferase (OsGST1)	X	–	0.0
	J013160C22	OsPTR family protein, peptide transporter	X	–	1e-114
	J013072F03	ABC-type (ATP-binding cassette) transport protein	–	X	1e-126
	J023056A15	ABC-type (ATP-binding cassette) transport protein	X	–	4e-12
	J023069G03	ABC-type (ATP-binding cassette) transport protein	X	–	0.0
	002-159-F08	Sugar transport protein (membrane protein - OsSTP)	X	–	0.0
Transcription factor	002-105-H07	OsNAC5	–	X	1e-39
Others	J075106H22	Nicotianamine synthase 3 (OsNAS3)	–	X	9e-13

Sequences were obtained from SSH experiments and differential expression was confirmed by quantitative RT-PCR. A complete list of all genes obtained from the SSH experiments is shown in Supplementary Table 1

The libraries from which the genes were identified (IR68144 and/or IR75862) are indicated

transporter, identified in our experiment, could play a role during rice grain filling. The rice *Os*MST6 protein, which shares 79.6% identity with *Os*MST3, was also shown to actively participate in assimilate unloading and sugar supply during seed development (Wang et al. 2008). There are no functional data regarding the *Os*STP sugar transporter, found in our experiments. Another sugar transporter, *Os*SUT1, plays an important role in the transport of assimilates along the entire long-distance pathway, from the flag leaf blade to the base of the filling grain, acting primarily in phloem loading of sucrose retrieved from the apoplasm along the transport pathway (Scofield et al. 2007). The sugar transporters *Os*SUT2 and *Os*MST5, were also shown to play a role during the early stage of seed development (Takeda et al. 2001).

We found higher expression during grain filling of a member of the peptide transporter family (*Os*PTR) and three members of the ATP-binding cassette (ABC) protein, which could be important for N and Fe allocation to the grain, respectively. Members of the PTR family transport dipeptides and tripeptides as well as amino acids and nitrate (Stacey et al. 2002). Transgenic plants expressing the *β*-glucuronidase gene, under the control of the *AtPTR1* promoter, showed evidence of expression in the vascular tissue throughout the plant, indicative of a role in long-distance transport (Dietrich et al. 2004). Moreover, the PTR family gene *Na*NTR1 of the carnivorous pitcher plant *Nepenthes alata* was expressed in phloem cells within pitchers, indicating that *Na*NTR1 may function in phloem loading of peptide nitrogen exported from the pitcher to sink organs in the plant (Schulze et al. 1999). According to Curie and Briat (2003), several of the transporters that would be expected to be present for the transport of Fe or chelated Fe in plants have not been detected. The ABC superfamily of transporters uses ATP hydrolysis for the transmembrane translocation of a large variety of molecules (including peptides), and could play a role in Fe transport in plants (Curie and Briat 2003). One ABC transporter from barley roots, IDI7, was shown to be Fe-regulated (Yamaguchi et al. 2002). Proteins from a related family of rice peptide transporters, the oligopeptide transporters family (OPT), were already shown to transport iron. The *Os*OPT1, *Os*OPT3 and *Os*OPT4 proteins can transport ferrous and/or ferric iron chelated to nicotianamine, a metal chelating tripeptide (Vasconcelos et al. 2008). Recently, it was shown that the *Arabidopsis* *At*OPT3 protein functions in metal homeostasis and movement of Fe to developing seeds (Stacey et al. 2008). Although from a distinct group of peptide transporters, the ABC transport proteins found in our work could also participate in metal homeostasis and movement of Fe to developing seeds. However, further studies are necessary to test this possibility.

One of the sequences found as up-regulated in R5 stage, *Os*NAS3, belongs to the nicotianamine synthase (NAS) family, which catalyzes the trimerization of *S*-adenosylmethionine to form the metal chelating compound nicotianamine (NA; Higuchi et al. 2001). The transport forms of Fe and Zn in the phloem are still unclear, and a role for amino acids as chelators of micronutrients in the phloem sap has been discussed by Grusak and DellaPenna (1999). Von Wirén et al. (1999) suggested that Fe and Zn are most likely chelated by NA during phloem transport. Inoue et al. (2003) showed that NAS genes are expressed in cells involved in long-distance transport of Fe. Tobacco plants overexpressing NAS have higher seed concentrations of Zn, Mn (Kim et al. 2005), Cu and Fe (Takahashi et al. 2003; Kim et al. 2005) than control plants. It has been suggested that the Fe content in rice grains could be increased by manipulating *Os*NAS3 expression, assuming that translocation of Fe into grains is mediated by NA (Nishizawa 2005).

Ten transcription factors were detected in our experiments, including *Os*NAC5 (Supplementary Table 1). The NAC protein family comprises a variety of plant proteins that are identifiable by the presence of a highly conserved N-terminal NAC domain. NAC is an acronym derived from the names of the three genes first described as containing the domain, namely NAM (*no apical meristem*), ATAF1,2 and CUC2 (*cup-shaped cotyledon*) (Souer et al. 1996; Aida et al. 1997). NAC proteins have been implicated in transcriptional control of a variety of plant processes. Roles of the NAC family genes include embryo and shoot meristem development, lateral root formation, auxin signaling, defense and abiotic stress response (Olsen et al. 2005). According to Fang et al. (2008), *Os*NAC5 and 6 belong to the stress-responsive NAC (*SNAC*) subfamily in rice. Expression of the *Os*NAC6 gene is induced by abiotic stresses, including cold, drought, high salinity and abscisic acid application (Ohnishi et al. 2005). Its expression is also induced by wounding and blast disease (Nakashima et al. 2007). Expression of the NAC family genes in senescing leaves has been reported by several groups (Guo et al. 2004; Lin and Wu 2004; Buchanan-Wollaston et al. 2005; Guo and Gan 2006). *At*NAP, a gene encoding a NAC family transcription factor, is closely associated with the senescence process in *Arabidopsis* rosette leaves, and the mutant phenotype can be restored to wild-type by its homolog from rice (*Os*NAP or ONAC058; Guo and Gan 2006). The ONAC058 gene also belongs to the *SNAC* subfamily in rice (Fang et al. 2008). The senescence-associated expression pattern of over 20 other NAC family members suggests a role of the NAC family genes in leaf senescence (Guo et al. 2004; Buchanan-Wollaston et al. 2005). Gpc-B1, a wheat quantitative trait locus, is involved in more efficient remobilization of amino acids, zinc, iron and manganese from

leaves to the grains (Uauy et al. 2006a; Distelfeld et al. 2007). This ancestral wild wheat allele encodes a NAC transcription factor (NAM-B1), a single gene with multiple pleiotropic effects that regulates senescence and increases nutrient remobilization. Reduction in mRNA levels of the multiple NAM homologs by RNA interference delayed senescence by more than 3 weeks and reduced wheat grain protein, zinc, and iron content by more than 30%. Phylogenetic analyses revealed that the closest rice protein to the NAM-B1 wheat protein is ONAC010 (Locus ID Os07g37920) (Uauy et al. 2006b). However, this gene does not correspond to the *Os*NAC5 gene identified in our experiments (Locus ID Os11g08210).

Several genes previously associated with senescence processes in rice have been identified in our experiment (Supplementary Table 1), including the genes encoding: Ubiquitin-conjugating enzyme, GDSL-motif lipase/hydrolase family protein, Lipid transfer protein and J023008A06 Senescence-associated protein (Sperotto et al. 2007; Sperotto et al. 2008). The higher expression of such genes in flag leaves during grain filling (R5) suggest that a senescence process is already occurring, even without leaf chlorosis, the most obvious signal of leaf senescence. The expression of a senescence marker gene (Staygreen gene, *Os*SGR, a chloroplast protein which regulates chlorophyll degradation by inducing LHCII disassembly through direct interaction; Park et al. 2007) was significantly higher ($P \leq 0.05$) during the grain filling stage (R5) relative to the panicle exertion stage (R3) in plants from both tested cultivars (Fig. 2), indicating the occurrence of an early senescence process, still without visual signs. It is possible that events occurring in this early stage could be affecting mineral remobilization. Chlorosis was visually perceived during the R7 stage, when the highest SGR expression was seen in flag leaves of both cultivars (Fig. 2), indicating a well-established senescence process.

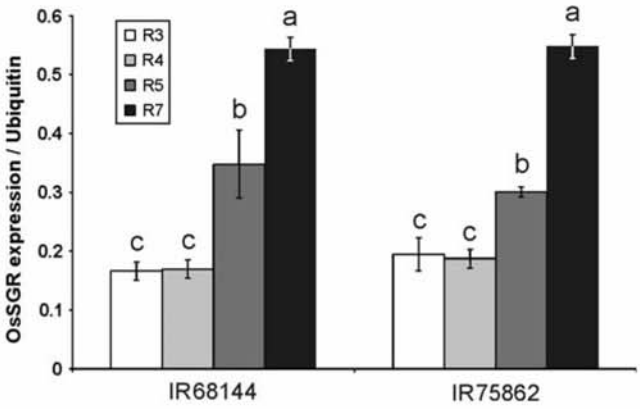

Fig. 2 Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Staygreen* (*Os*SGR) gene in flag leaves of *indica* IR68144 and *japonica* IR75862 rice cultivars. Flag leaves were collected in R3 (panicle exertion), R4 (anthesis), R5 (grain filling) and R7 (grain dry down) stages. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$)

Confirmation of subtractive cDNA expression patterns using qRT-PCR

To validate the SSH results and to confirm the up-regulated expression of the isolated genes, eight transporters (*Os*MST1, *Os*MST3, *Os*GST1, *Os*PTR, 3 ABC-type and *Os*STP), one sequence related to Fe homeostasis (*Os*NAS3) and one transcription factor (*Os*NAC5), all listed in Table 3, were further evaluated in flag leaves of four different rice cultivars (showing contrasting levels of Fe and Zn in the seeds) by quantitative RT-PCR, using an Ubiquitin gene as control. The differences in expression found by the SSH methodology were confirmed for the ten tested genes, with higher expression in R5 at least in the cultivar from which the sequence was identified (Fig. 3). However, the expression pattern of these genes varies among the four tested cultivars. *Os*MST3 and *Os*NAC5 were the only genes with up-regulated expression in R5 flag leaves relative to R3 in all tested cultivars. *Os*NAS3 and *Os*PTR expression was significantly higher in R5 of high iron cultivars (IR68144 and IR75862). Increased expression of the ABC-type transporter J013072F03 was seen in three of the four tested cultivars (Epagri 108, IR68144 and IR75862). The transcripts of *Os*STP were detected only during R5 in all tested cultivars (Fig. 3h). Expression of the *Os*MST3, *Os*ABC (J023056A15), *Os*ABC (J023069G03) and *Os*NAC5 genes was remarkably high (Fig. 3b, f, g, i), considering that their expression reached higher levels than Ubiquitin expression. Since NAC transcription factors have been described as associated with senescence processes (Guo et al. 2004; Buchanan-Wollaston et al. 2005) and nutrient remobilization (Uauy et al. 2006b), and considering that *Os*NAC5 increased expression during rice grain filling in all tested cultivars (Fig. 3i), we decided to further analyze this gene.

NAC domain analysis

The rice homolog with the highest amino acid sequence homology to wheat NAM-B1 is the ONAC010 gene (Locus ID Os07g37920). The amino acid sequences of the predicted NAC domain from the *Os*NAC5 and ONAC010 proteins share 65 and 86% identity, respectively, with the wheat NAM-B1 NAC domain. The same domains from the two rice proteins have 68% identical amino acids (Fig. 4). According to Fang et al. (2008), the rice NAC family can be classified into five groups (I–V), and phylogenetic analysis revealed that both *Os*NAC5 and ONAC010 belong to the group III (namely stress-responsive NAC genes,

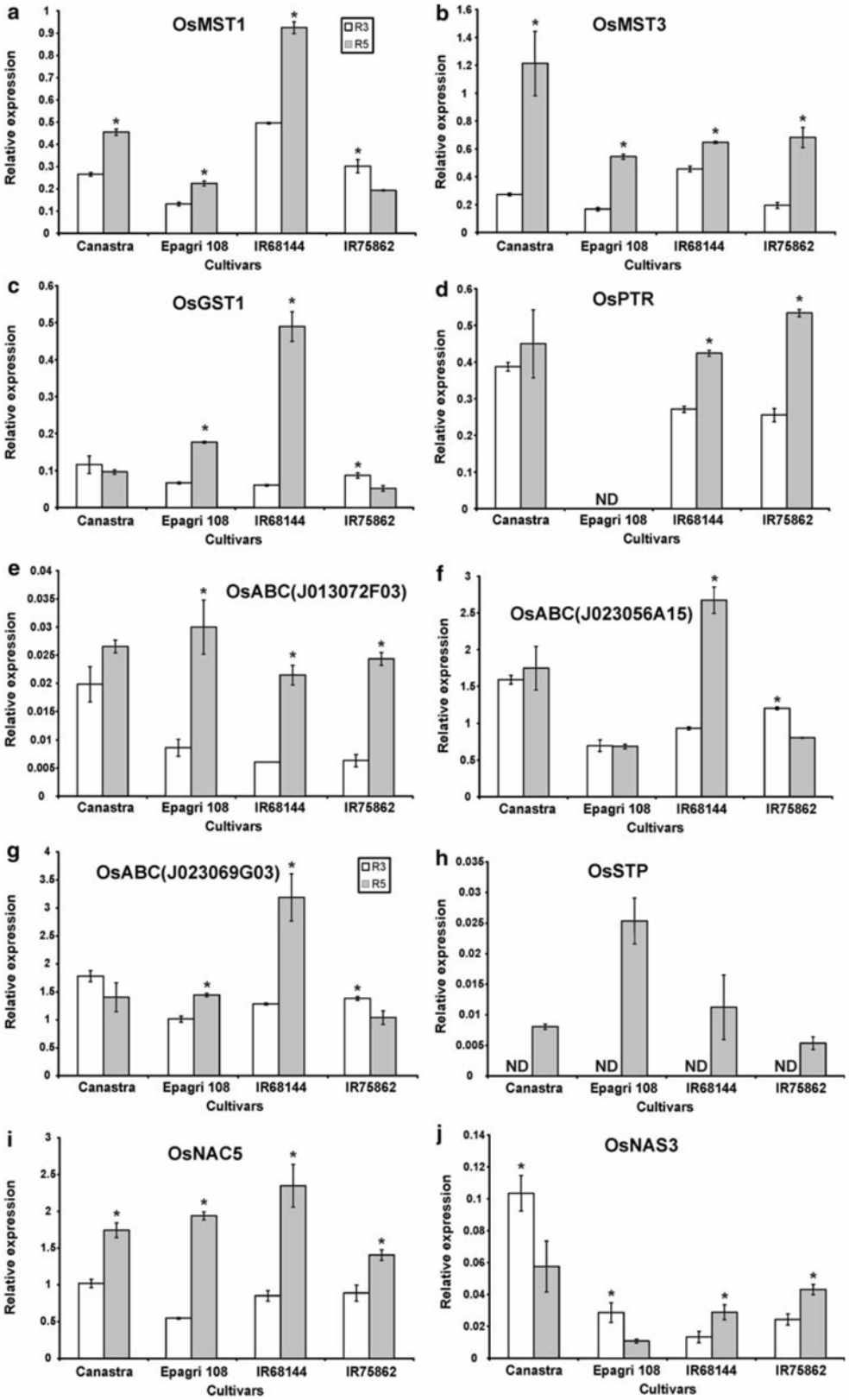

Fig. 3 Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of selected genes identified by SSH. Four diverse rice genotypes were used. Flag leaves were collected in R3 (panicle exertion) and R5 (grain filling) stages. *Os*MST1, monosaccharide transporter 1; *Os*MST3, monosaccharide transporter 3; *Os*GST1, glutathione-S-transferase 1; *Os*PTR, peptide transporter; *Os*ABC, ATP-binding cassette transport protein; *Os*STP, sugar transport protein; *Os*NAC5, NAC5 transcription factor; *Os*NAS3, nicotianamine synthase 3. Values are the averages of three samples ± SE. Mean values with one asterisk are different by *t* test ($P \leq 0.05$). *ND* not detected

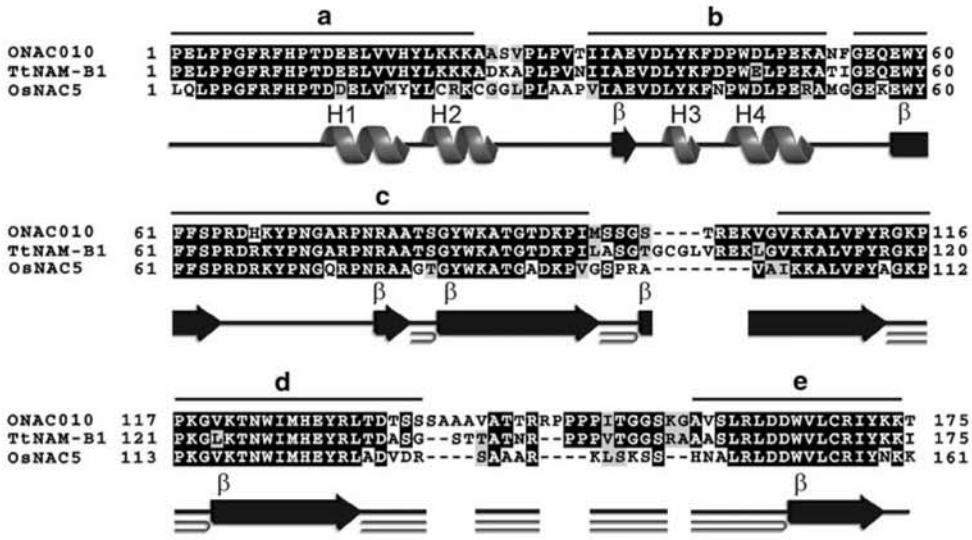

Fig. 4 Amino acid alignment of the NAC domain of ONAC010 (Os07g37920), *Tt*NAM-B1 (DQ869673) and *Os*NAC5 (Os11g08210). The five NAC conserved motifs are indicated by *black bars* a, b, c, d and e (Fang et al. 2008; Ganesan et al. 2008). The secondary structure of *Os*NAC5 is shown under the alignment. Letters indicate predicted helix (H) or beta sheaths (β). Alignment was performed using CLUSTALW and BOXSHADE; secondary structure was depicted from ProMotif analysis of PDB Sum

SNAC). Based on the composition of motifs found in the NAC domain, the rice NAC family can be classified into 15 types (A–O) (Fang et al. 2008; Ganesan et al. 2008). Both *Os*NAC5 and ONAC010 belong to group A and share a complete NAC DNA-binding domain (five motifs named a–e, as seen in Fig. 4) and a variable transcriptional regulation domain (Fang et al. 2008; Ganesan et al. 2008). We were able to detect low expression of ONAC010 in panicles and confirm the efficiency and specificity of the primers used. The ONAC010 gene was not expressed in R3 and R5 flag leaves from IR75862 rice plants (data not shown). According to Fang et al. (2008), ONAC010 is specifically expressed in stamen and induced by drought and salt. These data suggest that the rice closest homolog to the wheat NAM-B1 gene is not performing the same function as in wheat. Therefore, it is important to look for other potential NAC transcription factors in rice which may be affecting the senescence and nutrient remobilization processes.

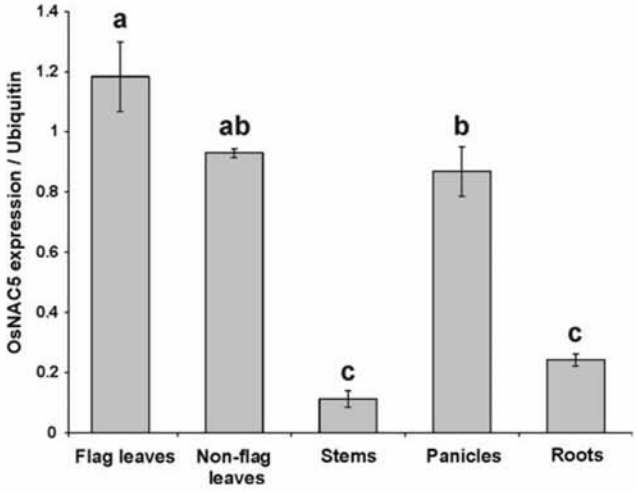

Fig. 5 Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*NAC5 in different rice organs collected during the R5 (grain filling) stage. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$)

*Os*NAC5 expression in different plant organs

The *Os*NAC5 gene was identified in the IR75862 library. Therefore, we used this cultivar for its further characterization. To elucidate the organ-specific expression of *Os*NAC5 gene, flag leaves, non-flag leaves, stems, panicles and roots were analyzed during the grain filling stage (Fig. 5). Accumulation of *Os*NAC5 mRNA is organ specific, with higher expression in flag leaves, non-flag leaves and panicles. Low levels of expression were seen in stems and roots. Transcripts of wheat NAM-B1 were also detected in flag leaves, spikes and peduncles after anthesis (Uauy et al. 2006b). Surprisingly, Kikuchi et al. (2000) showed that *Os*NAC5 was predominantly expressed in roots and embryos, with no detectable expression in leaf blades of mature plants and young panicles. A possible explanation for such differences in organ specificity could be the use of a different cultivar and of a completely different method for transcript detection (Southern blot analysis of PCR products) in their experiments.

*Os*NAC5 expression in flag leaves and panicles

The expression pattern of *Os*NAC5 gene was evaluated at different stages during the reproductive development of plants from four different rice cultivars (showing contrasting levels of Fe, Zn and protein in the grain). Figure 6a and b shows that the expression pattern of *Os*NAC5 in flag leaves and panicles varied among the cultivars. In Epagri 108 plants, which bear the lowest seed Zn and protein

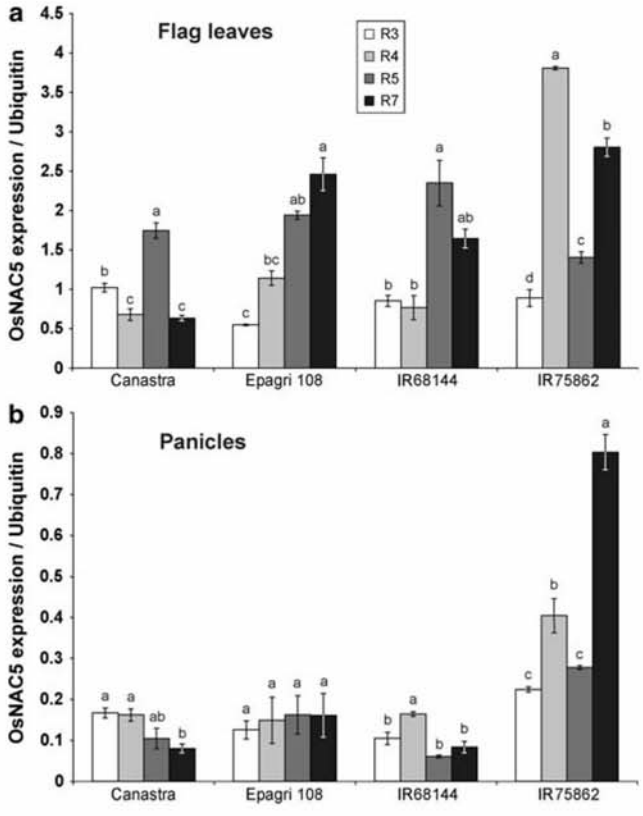

Fig. 6 Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*NAC5 in flag leaves (**a**) and panicles (**b**) of four diverse rice cultivars. Tissues were collected in R3 (panicle exertion), R4 (anthesis), R5 (grain filling) and R7 (grain dry down) stages. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$)

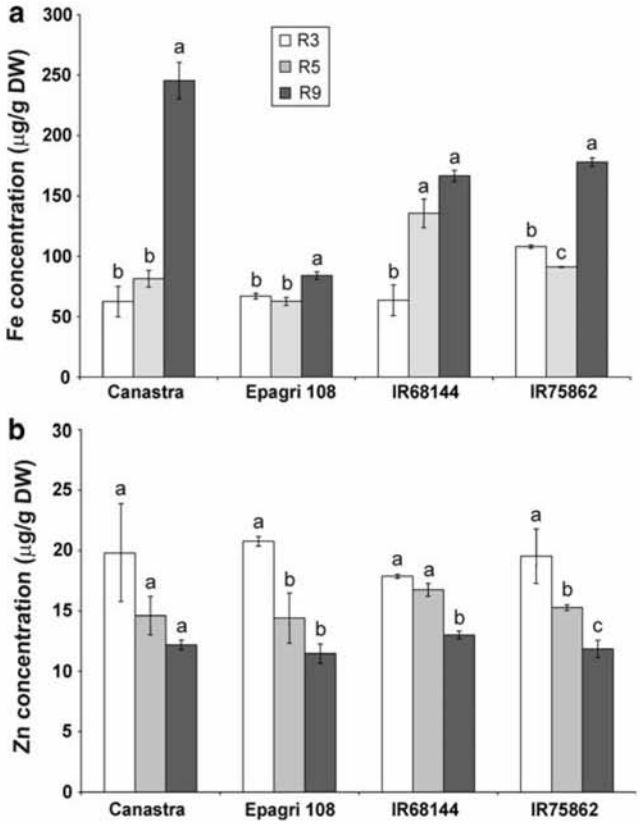

Fig. 7 Iron (**a**) and Zinc (**b**) concentrations in flag leaves from Canastra, Epagri 108, IR68144 and IR75862 rice cultivars. Flag leaves were collected at R3 (panicle exertion), R5 (grain filling) and R9 (full maturity) stages. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$). *DW* dry weight

concentrations of the cultivars studied, there was a gradual increase of *Os*NAC5 expression in flag leaves (highest expression in R7 stage) and constant expression levels in panicles. Very high *Os*NAC5 expression during R4 stage in flag leaves and during all stages in panicles (especially in R4 and R7) was seen in IR75862, which has the highest Fe, Zn and protein concentrations. It is possible that an earlier and higher increase in expression of *Os*NAC5 in flag leaves and panicles in the IR7582 cultivar may help to achieve higher Fe, Zn and protein concentrations in its seeds.

Elemental analysis of flag leaves

The only cultivar which showed a decrease in flag leaf Fe concentration in R5 relative to the R3 stage was IR75862 (Fig. 7a), which contains high Fe concentration in the grains and extremely high expression of *Os*NAC5 in flag leaves and panicles during the R4 stage. Such decrease in Fe concentration may indicate that at least a small portion of the pre-R5 stage Fe present in IR75862 flag leaves was mobilized to other organs, which could be the grains. It is interesting to note that Canastra and Epagri 108 cultivars (both with low Fe concentrations in the grain, around 10 ppm) appear to have different limiting steps to get Fe to the rice grain (Fig. 7a). Canastra has extremely high Fe concentration in flag leaves at the R9 stage (residual Fe, which will not be transported or mobilized). Moreover, Epagri 108 has the lowest Fe concentration in flag leaves during the R9 stage. However, we believe that it is not a result from high Fe mobilization, as it has already been shown that besides low grain Fe concentration, this cultivar has low Fe concentration in roots and especially in shoots, even in control conditions (Silveira et al. 2007). Our results suggest that low Fe concentration in rice seeds can be the result of low continued uptake and transport to the flag leaf (seen in Epagri 108 plants) or a low level of Fe export from the flag leaf (seen in Canastra plants). The extremely high Fe concentration in R9 flag leaves (Fig. 7a) and the low Fe concentration in seeds (Fig. 1a) of Canastra plants supports the concept that Fe mobilization through flag leaves is a potential rate-limiting step in the movement of Fe into developing grains (Waters and Grusak 2008).

The Zn concentrations decreased in flag leaves during reproductive development in all tested cultivars, except Canastra, indicating that Zn can be mobilized from flag leaves. IR75862 (which has the higher Zn concentration in seeds and the higher expression of *Os*NAC5 in the R4 stage) was the only cultivar with a consistent decrease in flag leaf Zn concentration from R3 to the R9 stage (Fig. 7b).

Nutrient remobilization to the grain has been linked to leaf senescence in wheat. The presence of the Gpc-B1 allele conferred earlier flag leaf senescence and it was suggested that the Gpc-B1 locus is involved in more efficient remobilization of amino acids, Zn, Fe and Mn from leaves to grains, in addition to its effect on earlier senescence of the green tissues (Uauy et al. 2006a; Distelfeld et al. 2007). These effects are caused by the NAM-B1 gene, located in the Gpc-B1 locus, which encodes a NAC domain-containing protein (Uauy et al. 2006b). In the absence of external stressors, initiation of senescence is dependent on age and developmental stage. Because the *Os*NAC5 gene could be a functional homolog of the NAM-B1 gene, we decided to investigate its expression during stress-induced senescence processes.

Expression of *Os*NAC5 during senescence

Although leaf senescence occurs in an age-dependent manner, the initiation and progression of senescence can be influenced by a variety of plant hormones and stress conditions. To test the possibility that *Os*NAC5 could be involved in stress-induced senescence, we detached leaves from IR75862 plants and kept them in the dark for 3 days at 27°C. Darkness is one of the most potent external stimuli for inducing leaf senescence (Kong et al. 2006). Leaf chlorosis was seen after 3 days of incubation (Fig. 8c), suggesting that senescence was already started, confirmed by the gradual increase of *Os*SGR expression (Fig. 8a). *Os*NAC5 expression also increased gradually (Fig. 8b). We also kept the detached leaves in the dark with 50 µM BAP (6-benzylaminopurine, a senescence inhibitor). There was either a minimal or no increase in *Os*SGR and *Os*NAC5 gene expression, and the leaves maintained the green color up to the third day of treatment (Fig. 8a–c). With the addition of 50 µM ABA (abscisic acid, a senescence inductor), leaves turned completely yellow in the third day, indicating a well-established senescence process. There was a gradual increase in *Os*SGR gene expression, but reaching higher levels relative to the dark treatment alone. In the first day of treatment with ABA, *Os*NAC5 expression was the same as in the third day of dark treatment (Fig. 8b). These data suggest that *Os*NAC5 could be involved in stress-induced senescence processes in addition to age-dependent senescence. The high increase in *Os*NAC5 gene expression seen

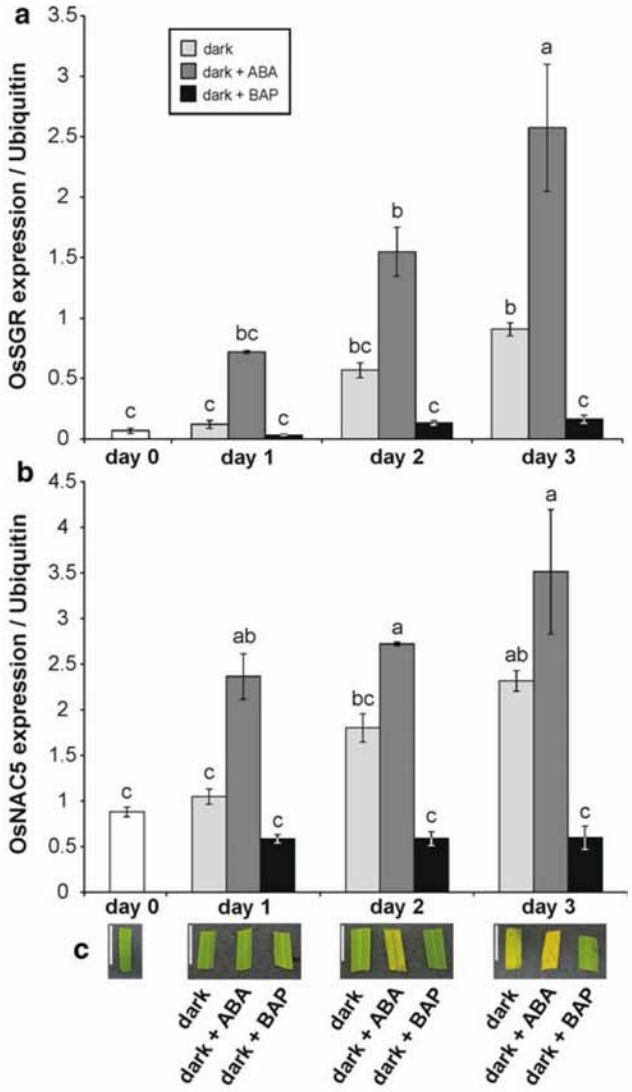

Fig. 8 Dark and hormonal regulation of leaf senescence in rice. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*SGR (**a**) and *Os*NAC5 (**b**) in detached leaves of IR75862 cultivar maintained for 3 days in the dark, dark + ABA or dark + BAP. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$). Each mean was compared with all the other means shown in the same figure. (**c**) Detached leaves of IR75862 cultivar. Bars in figures indicate 1 cm. *ABA* abscisic acid; *BAP* 6-benzylaminopurine

in the first day of treatment with ABA could be a response to abscisic acid itself. Evaluating the *cis*-elements present in the promoter region of the *Os*NAC5 gene might give us a better understanding of the involvement of ABA in *Os*NAC5 up-regulation. The 1,500 nucleotide region upstream of the transcription initiation site of this gene was analyzed using the database analysis tool from PLACE (Higo et al. 1999), with special attention given to potential abscisic acid-responsive elements. We found ten ABRELATERD1 (ACGTG) elements (Nakashima et al. 2006) (data not shown). This conserved element is involved in

abscisic acid signaling and is present in promoter regions of ABA-inducible genes (Abe et al. 2003). In order to test if the ABRELATERD1 elements were over-represented in *Os*NAC5 promoter, we analyzed 24,209 rice promoters spread through the 12 rice chromosomes (Morris et al. 2008, http://www.bioinformatics2.wsu.edu/Osiris) and we found an average of 2.31 ABRELATERD1 elements per promoter (data not shown). The presence of these motifs alone does not confirm that ABA regulates *Os*NAC5 expression, but suggests that its up-regulation during senescence could be ABA dependent.

ABA is known to play a major role in abiotic stress in plants (Leung and Giraudat 1998). Salt, cold and drought stresses are related to ABA production (Cheng et al. 2009). Therefore, we decided to test if *Os*NAC5 is up-regulated by stress conditions that lead to ABA accumulation. High salinity, cold and heat stresses were imposed to rice plants and *Os*NAC5 expression was evaluated in leaves. Increased transcript level of this gene was detected in fully expanded leaves of plants submitted to high salinity and cold stresses. Salt treatment resulted in increased *Os*NAC5 expression only after 4 days of treatment and cold treatment was found to slightly induce (without statistical significance) the expression of *Os*NAC5 after the same period of time (Fig. 9b). The higher expression of this gene after prolonged stress treatment suggests that *Os*NAC5 is up-regulated by stress-induced senescence, instead of directly by stress imposition. A well-established senescence process is occurring after 4 days of salt and cold stress, as confirmed by higher expression of *Os*SGR (Fig. 9a) and obvious stress symptoms, such as chlorosis and folded/twisted leaves (data not shown). *Os*NAC6, which belongs to the same subfamily of *Os*NAC5 (*SNAC*) is also induced by cold, high salinity and ABA application (Ohnishi et al. 2005). High temperature (40°C) did not cause any obvious sign of stress, and *Os*NAC5 and *Os*SGR gene expression were not up-regulated even after 4 days of treatment. According to Fang et al. (2008), there are 140 putative NAC or NAC-like genes in the rice genome. Twenty of these genes were identified as stress-responsive genes, including *Os*NAC5. However, we believe that its stress-induced expression is triggered only after the initiation of a stress-induced senescence process, instead of being triggered directly by the stress itself.

Since salt treatment resulted in the highest levels of *Os*NAC5 expression, we decided to use this stress treatment to test whether *Os*NAC5 up-regulation is ABA dependent. When plants were treated with 100 mM NaCl + 50 mM nicotinamide (a known inhibitor of ABA-mediated responses; Leckie et al. 1998), no increase in *Os*NAC5 expression could be detected (Fig. 10b), strongly suggesting an ABA-dependent regulation. A similar expression pattern was observed for the *Os*SGR gene, suggesting a close relationship between the two genes (Fig. 10a).

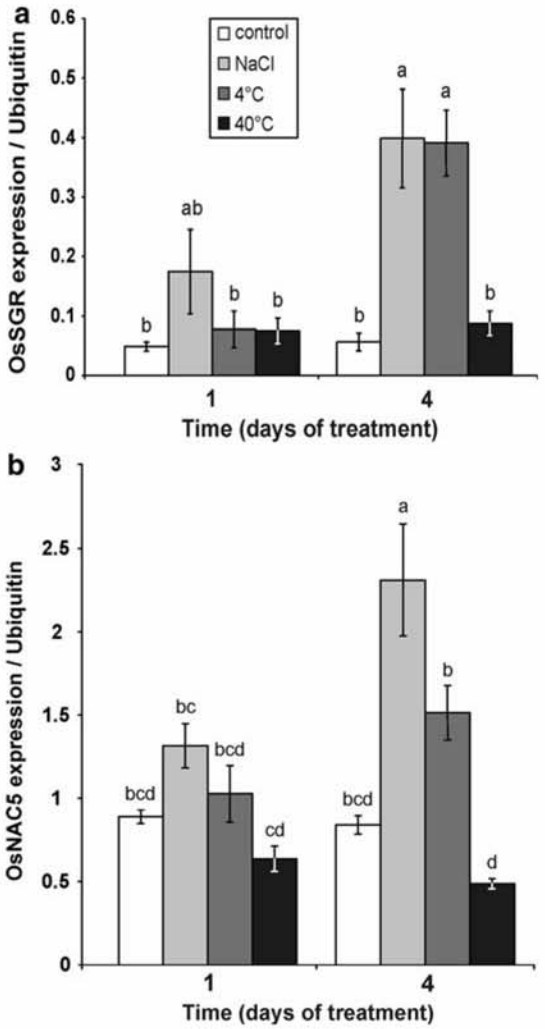

Fig. 9 *Os*NAC5 expression after stress imposition. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*SGR (**a**) and *Os*NAC5 (**b**) in fully expanded leaves of IR75862 rice plants. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$). Each mean was compared with all the other mean values shown in the same figure

*Os*NAC5 is not up-regulated by Fe-deficiency in rice leaves

Using microarray analysis, Ogo et al. (2006) showed up-regulation of *Os*NAC5 expression in Nipponbare rice shoots after short exposure to Fe-deficiency. In order to test the hypothesis that *Os*NAC5 is up-regulated by Fe-deficiency-induced senescence instead of directly by Fe-deficiency, we conducted a real-time PCR analysis. According to our results, *Os*NAC5 expression is not up-regulated by Fe-deficiency in rice leaves in the first days of treatment, neither in IR75862 (Fig. 11a) nor in Nipponbare plants (data not shown), as previously reported (Ogo et al. 2006). The up-regulation of this gene starts only at the beginning of Fe-deficiency-induced senescence of rice leaves

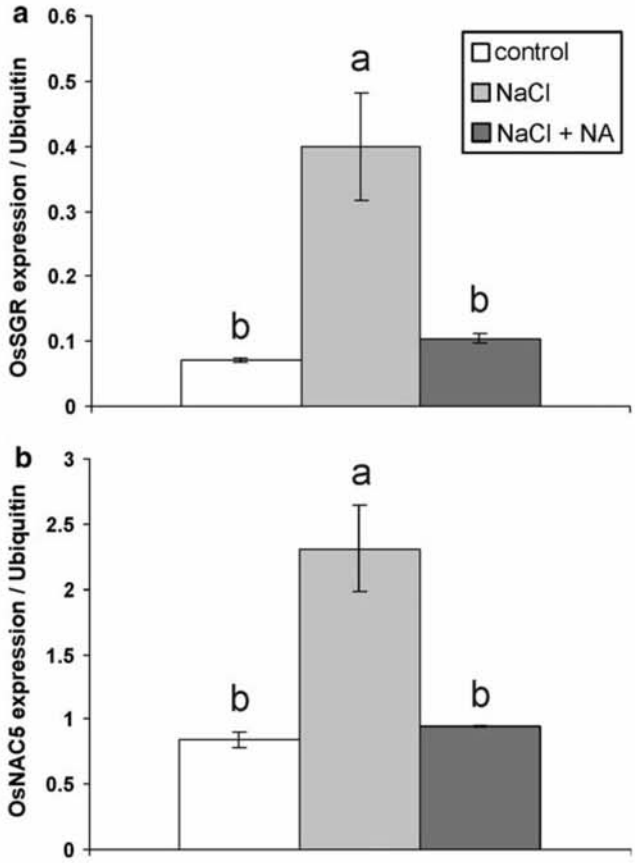

Fig. 10 *Os*NAC5 expression is ABA dependent. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*SGR (**a**) and *Os*NAC5 (**b**) in fully expanded leaves of IR75862 rice plants after 4 days of treatments. Values are the averages of three samples ± SE. Different letters indicate that the mean values are different by the Tukey HSD test ($P \leq 0.05$). *NA* nicotinamide. *Error bars* may be too small to be visible in the figure

(Fig. 11a), when higher expression of *Os*SGR gene is also observed (Fig. 11b). Expression analysis of *Os*IRO2 (Ogo et al. 2006) was used to confirm that plants were under Fe-deficiency (Fig. 11c). A possible explanation for the differences in gene expression between our work and the results from Ogo et al. (2006) could be the plant organs analyzed. We used only fully expanded leaves, and they used entire rice shoots. Another explanation would be the completely different nature of the used techniques (quantitative RT-PCR versus microarray). Although quantitative PCR will never generate the large amount of data that is achieved by cDNA microarrays, PCR has the advantage of unparalleled sensitivity (Schmittgen and Livak 2008).

Possible relation between *Os*NAC5 expression in flag leaves and Fe, Zn and protein concentration in seeds

To search for relationships between *Os*NAC5 expression at different stages of reproductive development in flag leaves and final mineral and protein concentrations in seeds, Pearson's correlation analyses were performed for different rice cultivars. No significant correlations were found for Cu, Mn and Mo (data not shown). Surprisingly, there were positive correlations between *Os*NAC5 expression in R3 (panicle exertion) and R4 (anthesis) stages with final seed Fe, Zn and protein concentrations (Table 4), instead of during R5 (grain filling stage, when most mineral remobilization was expected to occur). However, the extreme data point for IR75862 cultivar in R4 stage (Fig. 6a) could be driving most of the correlation found in this stage. Moreover, a larger number of rice cultivars with contrasting levels of Fe, Zn and protein in the seeds will need to be tested before this trend can be confirmed.

The importance of mineral remobilization in early stages of reproductive growth has been demonstrated in wheat. Expression of the NAM-B1 gene, an ancestral allele of a NAC transcription factor, is responsible for the earlier onset of flag leaf senescence, resulting in more efficient remobilization of protein, Zn, Fe and Mn from leaves to the grains (Distelfeld et al. 2007). NAM-B1 expression is seen right after anthesis (Uauy et al. 2006b). Moreover, the highest seed Zn concentration following foliar Zn applications in wheat was found at the beginning of seed development (around 10 days after anthesis or around the early milky stage), suggesting that applying foliar Zn during early stages of wheat reproductive development could be an effective way of increasing seed Zn concentration through efficient flag leaf export to the grain (Ozturk et al. 2006). Our results showed a correlation between Fe, Zn and protein concentrations in rice seeds (Table 2). Thus, simultaneous early mobilization of Fe, Zn and amino acids to developing seeds is not unexpected. Indeed, it has been shown that high Zn accumulation during early seed development is possibly related to protein synthesis. There are several reports showing that protein synthesis in seeds is particularly high during early seed development (Greene 1983; Martre et al. 2003) and Zn is the most critical micronutrient affecting protein synthesis in plants (Obata et al. 1999). A strong correlation between wheat grain Zn and grain protein was also shown previously (Morgounov et al. 2007), indicating that grain protein may be a sink for Zn. Iron is also remobilized from vegetative organs to the seed (Hocking and Pate 1977), and known Fe transporters were shown to be able to transport zinc as well (Guerinot 2000; Schaaf et al. 2005), which could explain, at least partially, simultaneous allocation of both minerals. Using a small set of rice cultivars, we also found a correlation between *Os*NAC5 expression in early stages of flag leaf development and Fe, Zn and protein concentrations in seeds (Table 4). Therefore, it is possible that *Os*NAC5 expression in flag leaves during the early stages of the grain filling process has a positive influence on Fe, Zn and amino acid transport from flag leaves to panicles.

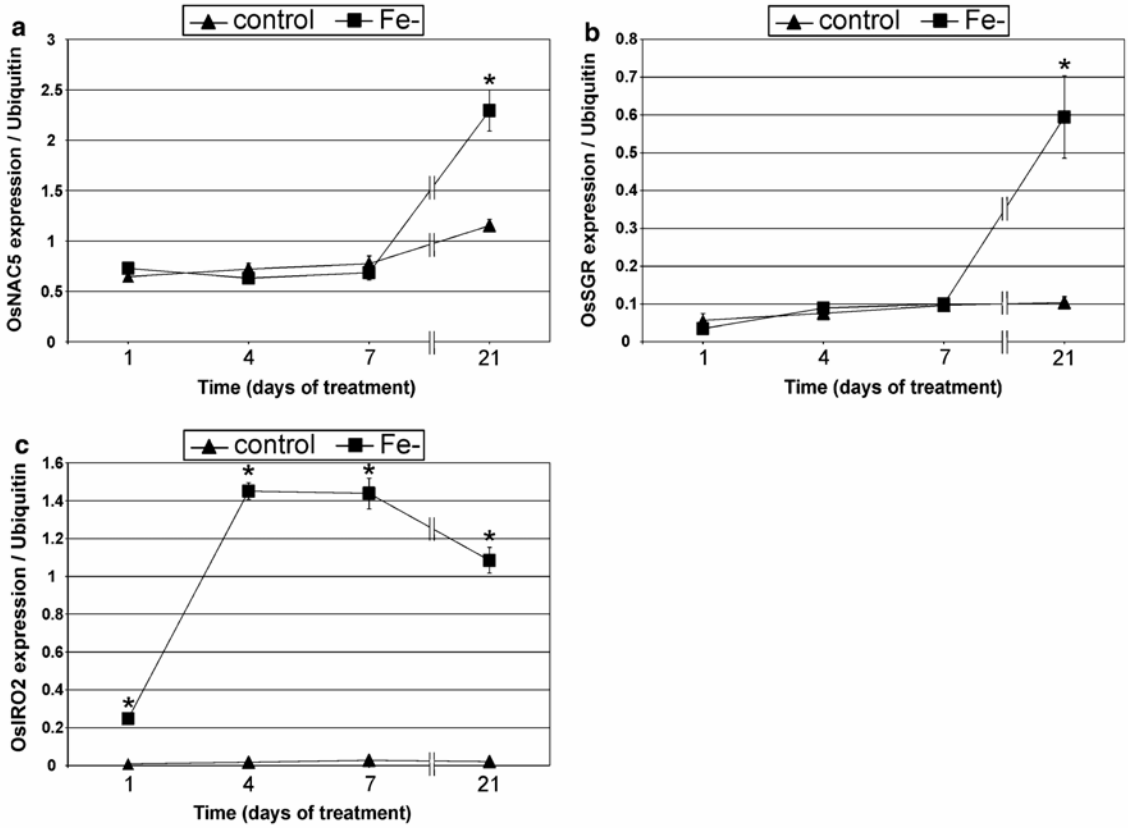

Fig. 11 *Os*NAC5 expression is not up-regulated by Fe-deficiency. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*NAC5 (**a**), *Os*SGR (**b**) and *Os*IRO2 (**c**) in fully expanded leaves from IR75862 rice plants. Values are the averages of three samples ± SE. Mean values with one *asterisk* are different by *t* test ($P \leq 0.05$). Some *error bars* do not extend beyond symbols

Table 4 Correlation analyses between *Os*NAC5 expression in flag leaves during the reproductive development and Fe, Zn and protein concentrations in rice seeds

	Fe	Zn	Protein
*Os*NAC5-R3	0.705**	0.843**	0.657**
*Os*NAC5-R4	0.731*	0.854**	0.750*
*Os*NAC5-R5	−0.021	−0.108	0.254
*Os*NAC5-R7	0.566	0.348	0.441

Analyses were performed with data from eight rice cultivars (Canastra, Epagri 108, BR-IRGA421, BR-IRGA409, IR68144, IR68144_1, IR69428 and IR75862, expression data shown in Supplementary Fig. 2) in stages R3 and R5 and from four rice cultivars (Canastra, Epagri 108, IR68144 and IR75862) in stages R4 and R7

* Significant at 0.05 probability level; ** significant at 0.01 probability level

Investigations are underway using a T-DNA mutant and overexpressing lines of *Os*NAC5 to clarify the involvement of this gene in rice grain filling. These lines should help clarify the existence of any relationship between the role of *Os*NAC5 in senescence and its possible influence on nutrient allocation to seeds.

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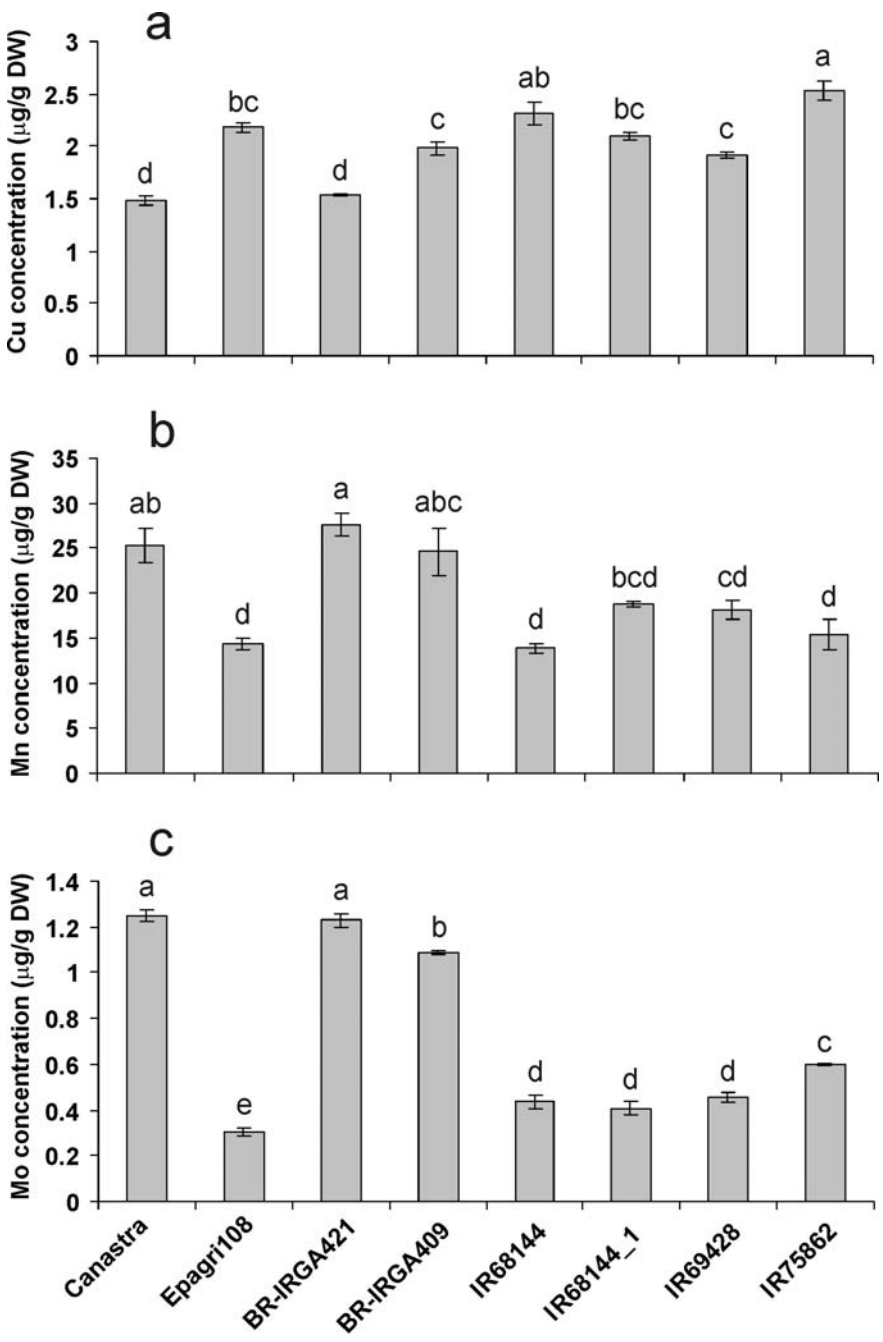
Supplementary Table 1. Up-regulated genes in rice flag leaves during the grain filling (R5) stage.

Functional categories	Rice Pipeline clone number	Description	IR68144 (*indica*)	IR75862 (*japonica*)	E-value
Transport	J013003K16	**Monosaccharide transporter 1 (OsMST1)**	X	-	0.0
	J033117G08	**Monosaccharide transporter 3 (OsMST3)**	-	X	1e-108
	001-123-F01	**Glutathione-S-transferase (OsGST1)**	X	-	0.0
	002-159-F08	**Sugar transport protein (membrane protein - OsSTP)**	X	-	0.0
	J013160C22	**OsPTR family protein, peptide transporter**	X	-	1e-114
	J013072F03	**ABC-type (ATP-binding cassette) transport protein**	-	X	1e-126
	J023056A15	**ABC-type (ATP-binding cassette) transport protein**	X	-	4e-12
	J023069G03	**ABC-type (ATP-binding cassette) transport protein**	X	-	0.0
	J090046E17	ATP-synthase beta subunit (atpB)	X	X	0.0
	J090091L04	H^+-transporting two-sector ATPase chain a	X	X	1e-147
	J090082A07	Chloroplast envelope membrane protein, proton extrusion protein-related (potential heme-binding protein)	X	X	0.0
Transcription factors	J013029P13	MYB family transcription factor	X	-	6e-16
	J090053J23	MYB family transcription factor	-	X	1e-135
	J023150J15	Zinc finger (C3HC4-type RING finger) protein	-	X	3e-89
	J023028G02	Zinc finger (C3HC4-type RING finger) protein	X	-	1e-95
	J013050D10	Zinc finger CCHC domain-containing	X	-	2e-16
	J033048I07	Protease-associated zinc finger (C3HC4-type RING finger)	X	-	0.0
	J013097L22	DNA-binding bromodomain-containing protein	-	X	0.0
	001-014-E08	Chitin-inducible gibberellin-responsive protein (putative SCARECROW gene regulator)	X	-	1e-140
	J023099N07	Probable RNA-binding protein	-	X	6e-97
	002-105-H07	**OsNAC5**	-	X	1e-39
Photosynthesis and photorespiration	001-200-A05	Ribulose-1,5-bisphosphate carboxilase oxygenase large subunit	X	X	1e-168
	J013001C24	Ribulose-1,5-bisphosphate carboxilase oxygenase activase	X	-	6e-25
	J090053P07	Photosystem II D2 protein	X	X	0.0
	J023063I15	Photosystem II 10 KDa protein	X	-	0.0
	001-100-G11	Photosystem I P700 apoprotein A1	-	X	1e-160
	J013002H23	Protochlorophyllide reductase	-	X	1e-115
	J075022M24	Plastocyanin	-	X	4e-98
	J100024A13	Blue copper-binding protein (plastocyanin-like domain)	X	-	6e-11
	002-112-G02	Cytochrome b6	X	-	0.0
	002-111-H11	Cytochrome c oxidase subunit 1 (cox1)	X	X	1e-162
	J033068O18	Fructose-1,5-bisphosphate aldolase	X	X	0.0
	J090019C07	NADPH-plastoquinone oxireductase subunit K	X	X	1e-89
	J100061C07	NADPH-plastoquinone oxireductase chain 3	X	X	0.0
	J023057C24	Fructose-1,6-bisphosphatase (cytosolic isoform)	X	X	1e-110
	J013052N09	Glycolate oxidase	X	-	5e-93
	006-204-B09	Glycine dehydrogenase	-	X	1e-134
Protein biosynthesis	J090052J10	Ribosomal protein S3	X	X	0.0
	J013084G06	Ribosomal protein L16	-	X	1e-121
	J013001E24	Ribosomal protein 41	-	X	6e-88

	J090097N04	Ribosomal protein S7	-	X	0.0
Protein modification	J023110H06	Phosphatidic acid phosphatase-related (PAP2-related)	X	-	8e-41
	J013020G01	Serine/threonine protein kinase	X	-	2e-38
	J023028J20	Prolyl endopeptidase (post-proline cleaving enzyme)	-	X	2e-88
	J013025E12	Mitochondrial-processing peptidase beta subunit (metalloendopeptidase)	-	X	1e-149
Protein degradation	J033054B21	Ubiquitin-conjugating enzyme	-	X	1e-113
Structural	J013033M18	β-tubulin	-	X	1e-177
Lipid metabolism	001-124-B12	GDSL-motif lipase/hydrolase family protein	X	X	1e-164
	J090063J18	Lipid transfer protein (LTP) family protein	-	X	1e-85
	006-301-H11	Phospholipid/glycerol acyltransferase family protein	X	-	3e-12
Others	001-201-C12	Transcription initiation factor sigma 5 (SIG5), plastid-specific	X	X	1e-62
	001-201-C07	Adenosine monophosphate-binding protein 3 (AMPBP3)	X	X	1e-148
	J065163N20	Ethylene-insensitive 3-like protein (OsEIL1)	X	-	9e-53
	001-202-B04	Actin-depolymerizing factor	-	X	0.0
	J013056J05	SPX (SYG1/Pho81/XPR1) domain-containing protein	X	-	2e-63
	J090034J17	DNA-directed RNA polymerase beta chain (plastid-encoded)	X	X	0.0
	J075133L10	SPL1-related protein	-	X	6e-20
	J033073A13	Cullin-like protein	-	X	8e-38
	J075106H22	**Nicotianamine synthase 3 (OsNAS3)**	-	X	9e-13
	J013108J23	NADH-dependent hydroxypyruvate reductase	-	X	0.0
	006-204-A03	Senescence-associated protein	X	-	0.0
Unknown	001-030-H01	Hypothetycal 12K chloroplast protein	X	X	0.0
	J023008A06	Unknown senescence-associated protein	-	X	0.0
	006-212-D12	Unknown protein	X	X	0.0
	001-203-E01	Unknown protein	X	X	0.0
	J090050F01	Unknown protein	-	X	0.0
	J100039M09	Unknown protein	X	X	9e-84
	J075092B19	Unknown protein	X	-	1e-104
	006-209-B02	Unknown protein	X	X	0.0
	001-034-D12	Unknown protein	X	-	2e-20
	002-169-H11	Unknown protein	X	-	0.0
	001-008-E03	Unknown protein	X	-	0.0
	006-206-C02	Unknown protein	X	-	0.0
	006-309-F01	Unknown protein	-	X	7e-93
	002-143-D06	Unknown protein	-	X	1e-88
	J013033G18	Unknown protein	-	X	6e-94
	002-150-C09	Unknown protein	-	X	1e-125
	J013068G16	Unknown protein	-	X	0.0
	Total	78 [a]	47	51	

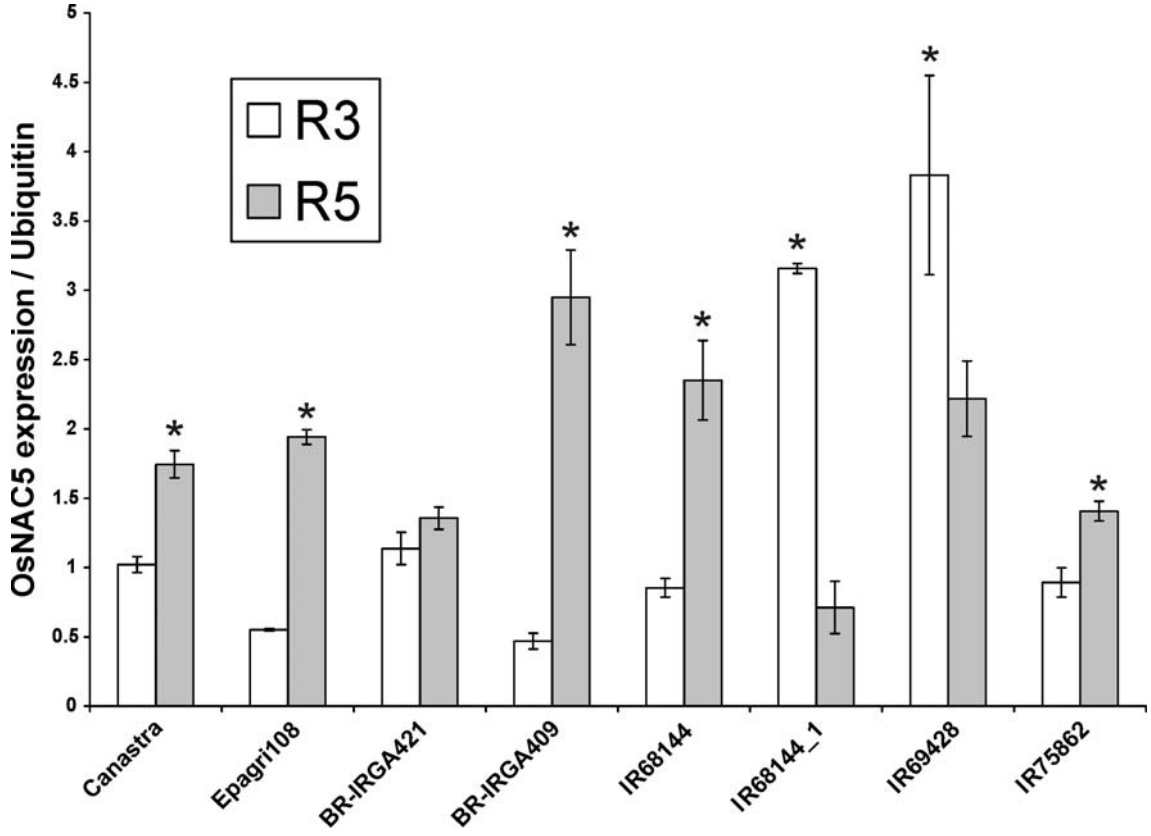
[a] Total number of different genes with homology to genes available in public databases.
Genes with names in bold were further analyzed by quantitative RT-PCR.
The libraries from which the genes were identified (IR68144 and/ or IR75862) are indicated.

Supplementary Figure 1



Supplementary Fig. 1 Copper (a), Manganese (b) and Molybdenum (c) concentrations in whole seeds from eight diverse rice cultivars. Seeds were collected at full maturity (R9 stage). Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). DW = dry weight.

Supplementary Figure 2



Supplementary Fig. 2 Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of *Os*NAC5 in flag leaves of eight diverse rice genotypes collected in R3 (panicle exertion) and R5 (grain filling) stages. Values are the averages of three samples ± SE. Means with one asterisk are different by *t* test ($P \leq 0.05$).

Capítulo 2

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Identification of putative target genes to manipulate Fe and Zn concentrations in rice grains

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ABSTRACT

Rice is the staple food of half of the world's population; however, it is a poor source of essential micronutrients such as Fe and Zn. Since flag leaves are one of the sources of remobilized metals for developing seeds, the identification of the molecular players that might contribute to the process of metal transport from flag leaves to the seeds may be useful for biofortification purposes. We analyzed the expression of 25 metal-related genes from rice, including rice homologues for *YSLs*, *NRAMPs*, *ZIPs*, *IRT1*, *VIT1* (coding for known or potential metal transporters), as well as *NASs*, *FROs* and *NAC5* (involved in metal homeostasis) in flag leaves of eight rice cultivars (showing contrasting levels of seed Fe and Zn) during panicle emergence (R3) and grain filling stage (R5). The expression level of nine of these genes (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*, *OsNAS1*, *OsFRO1* and *OsNAC5*) in flag leaves exhibited significant correlations with Fe and/or Zn concentrations in the seeds. In this way, our study has provided a short list of putative target genes to manipulate Fe and Zn concentrations in rice grains.

Introduction

Rice is the primary or secondary staple food for 50% of the world's population. Therefore, it is one of the most important crop plants on Earth (Lucca et al., 2002). However, rice is a poor source of essential micronutrients such as Fe and Zn (Bouis and Welch, 2010). Micronutrient malnutrition, and particularly Fe and Zn deficiencies, affect over three billion people worldwide, mostly in developing countries (Welch and Graham, 2004). Biofortification has emerged as one possible solution to alleviate malnutrition and the development of new cultivars with elevated concentrations of Fe and Zn would be extremely relevant (Zimmermann and Hurrell, 2002). However, the lack of knowledge about how nutrients are translocated from vegetative tissues to the seeds is one of the barriers to biofortification (Colangelo and Guerinot, 2006), resulting in uncertainty about the best genes or pathways to target for modification. In this way, a better understanding of metal homeostasis and localization, as well as the identification of the molecular players that might contribute to the process of metal transport to the seeds is essential.

Around 75% of total grain Zn was reported to be present in the endosperm of brown rice (Jiang et al., 2008), while X-ray fluorescence imaging revealed that Zn is most abundant in the embryo and in the aleurone layer (Takahashi et al., 2009). Fe has been localized by histochemical techniques in the aleurone layer and in the embryo, but not in the endosperm of non-transformed rice seeds (Prom-u-thai et al., 2003; Sivaprakash et al., 2006; Sellappan et al., 2009). X-ray fluorescence imaging allowed the identification of Fe also in the endosperm (Takahashi et al., 2009). Although reported to be present in protein bodies of aleurone cells and in phytin granules both in the aleurone and in the embryo's scutellar cells (Krishnan et al., 2001), there is no clear information in the literature about the prevalent form of Fe (ferric or ferrous) present in the rice seed. In this work, we used staining techniques to investigate the distribution and relative abundance of both Fe forms in a set of genotypes, trying to identify specific distribution patterns characteristic of those genotypes with higher seed Fe concentrations.

Several transporters potentially involved in metal ion homeostasis have been identified in the rice genome (Bughio et al., 2002; Gross et al., 2003; Ramesh et al., 2003; Koike et al., 2004). Most of

Abbreviations: DMA, deoxymugineic acid; FRO, ferric reductase oxidase; ICP-OES, inductively coupled plasma optical emission spectroscopy; IRT, iron-regulated transporter; NA, nicotianamine; NAC, NAC transcription factor; NAS, nicotianamine synthase; NRAMP, natural resistance-associated macrophage protein; VIT, vacuolar iron transporter; YSL, yellow stripe-like; ZIP, Zrt/Irt-like protein.

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these metal transporters are capable of transporting one or several divalent cations including Fe^{2+}, Zn^{2+}, Mn^{2+} and Cu^{2+} (Narayanan et al., 2007). However, most of these genes belong to gene families and the specific function of several of these transporters remains unknown. Furthermore, little information is available about how metals are transported to rice seeds and which of the processes (uptake from the soil, transport from the roots to the shoots, phloem loading, grain filling) are the rate-limiting steps for metals to reach the grain. This information, together with the identification of the genes which actively participate in each one of these processes, is essential to develop rice cultivars with improved nutrient concentrations.

Flag leaves are the major source of phloem-delivered photoassimilates for rice developing seeds, and are believed to be one of the sources of remobilized metals for the seeds (Narayanan et al., 2007; Sperotto et al., 2009). In another cereal (wheat), Zn and Fe remobilization from flag leaves to seeds was clearly demonstrated (Uauy et al., 2006; Waters et al., 2009). Our group has previously shown that flag leaf Zn concentration decreases during reproductive development in rice cultivars with high seed Zn concentrations (Sperotto et al., 2009). In the same work, flag leaf Fe concentrations decreased during reproductive development in a rice cultivar with high Fe concentration in seeds, while a cultivar with low seed Fe concentration showed a high level of residual Fe in flag leaves at reproductive maturity. Another group has presented evidence for net Zn remobilization from rice leaves and transport to the grain when the plants were challenged with reduced levels of root-supplied Zn during grain fill (Jiang et al., 2008). Together, these studies support the concept that at least some of the rice grain's Zn and Fe are mobilized from flag leaves and suggest that the relative contribution of the flag leaf may vary across different genotypes.

It is possible that other mechanisms of mineral allocation to the rice grain, such as remobilization from other leaves, phloem delivery of minerals acquired from the xylem through a transfer process or even direct xylem loading, may contribute with higher Fe and Zn amounts than flag leaf remobilization during grain filling in rice. However, significant increases in flag leaf remobilization could have a positive impact in biofortification efforts. Enhanced flag leaf remobilization could be achieved in the future, provided that the relevant genes and gene products are identified and studied. As we were interested in identifying genes that could be targets for improved Fe and Zn mobilization to seeds, we analyzed gene expression of 25 metal-related genes in flag leaves of eight different rice cultivars (showing contrasting levels of Fe and Zn in the seeds) during the period of panicle emergence and grain filling, which correspond to R3 and R5 stage, according to Counce et al. (2000). Furthermore, we correlated the expression level of these genes with final Fe and Zn concentrations in the seeds and were able to detect putative target genes to be used in conventional breeding or plant biotechnology to improve Fe and/or Zn concentrations in rice grains.

Materials and methods

Plant growth conditions

Plants from eight rice (*Oryza sativa* L.) cultivars [Canastra, BR-IRGA421, BR-IRGA409, IR68144 and IR68144_1 (five *indica* cultivars) and IR75862_1, IR75862 and IR69428 (three *japonica* cultivars)] were grown in soil under flooded conditions in the experimental unit of Instituto Rio-Grandense do Arroz (IRGA), in Cachoeirinha, RS, Brazil (29°54′58.61″S, 51°10′02.65″W), during the rice growing season (October 2007 to March 2008). Soil characteristics of this site were reported by Stein et al. (2009a). Entire flag leaf blades were collected during R3 (panicle emergence) and R5 (grain filling) stages (Counce et al., 2000), and immediately frozen in liquid nitrogen. Three samples were collected from a plant with multiple tillers and samples were pooled. Three biological replicates (independent plants) were collected for quantitative PCR studies. Seeds were also harvested from plants grown to full maturity (R9, according to Counce et al., 2000) for histochemical localization of Fe forms and for Fe and Zn quantification.

Iron localization in rice grains

Presence and localization of Fe was determined on rice grains of six genotypes (Canastra, BR-IRGA421, BR-IRGA409, IR68144_1, IR75862 and IR69428) with Prussian's and Turnbull's Blue histochemical reactions (Lillie, 1965). Prussian Blue reaction detects ferric iron (Fe^{3+}) through the treatment of samples with potassium ferrocyanide [$K_4Fe(CN)_6{\cdot}3H_2O$] acid solution (100 mg of ferrocyanide diluted in 10 ml of HCl 0.06 M), while Turnbull's Blue reaction localizes Fe in the ferrous form (Fe^{2+}) by exposing the samples to potassium ferricyanide [$K_3Fe(CN)_6$] acid solution (100 mg of ferricyanide diluted in 10 ml of HCl 0.06 M). Iron molecules present in the tissue result in a blue pigment in both reactions (Lillie, 1965). Grains of the six cultivars were exposed to 60 °C overnight to avoid germination and then soaked for 12 h in distilled water for free-hand sectioning. Grains were sectioned in half before being exposed to the solutions. Samples (14 half seeds per genotype) were kept in the solution for a minimum time of 1 h and a maximum of 1 h and 30 min for both reactions. The aleurone layer was evaluated on the external view, while endosperm, embryo and subaleurone layer were evaluated in the longitudinal internal section of the grain. Observations of rice grains were made in a Wild Heerbrugg stereoscopic microscope and documentation with a Kodak C-140 under bright-field mode.

Fe and Zn quantification by ICP

Fe and Zn concentrations were determined on rice grains of eight genotypes (Canastra, BR-IRGA421, IR68144, BR-IRGA409, IR75862_1, IR75862, IR68144_1 and IR69428). Whole seeds from nine plants were harvested and dried in a 60 °C oven for 48 h. Dried tissues were digested according to Narayanan et al. (2007). Fe and Zn quantification was performed using inductively coupled plasma optical emission spectroscopy (ICP-OES - CIROS ICP Model FCE12; Spectro, Kleve, Germany). Tomato leaves and rice flour standards (SRM 1573A and 1568A, respectively; National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the rice samples to ensure accuracy of the instrument calibration.

RNA extraction and cDNA synthesis

Total RNA was extracted using Concert Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). cDNA was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Mountain View, CA, USA), according to the manufacturer's instructions, in the presence of RNase OUT (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was performed with reverse transcriptase (M-MLV, Invitrogen, Carlsbad, CA, USA), using 1 μg of RNA.

Quantitative RT-PCR and data analysis

qRT-PCRs were carried out in an Applied-Biosystem 7500 real-time cycler. All primers (listed in Supplementary Table 1) were designed to have similar T_m values (60 ± 2 °C). Reaction settings were performed according to Sperotto et al. (2009). Gene expression was evaluated by the $2^{-\Delta C_T}$ method (Livak and Schmittgen,

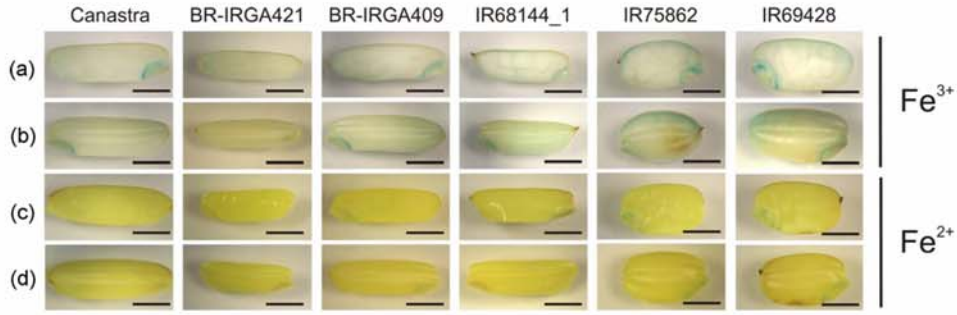

Fig. 1. Histochemical localization of ferric Fe through Prussian's reactions (a and b) and of ferrous Fe through Turnbull's Blue reactions (c and d) in six rice cultivars. External view of rice grains (b and d) and longitudinal internal sections (a and c) are shown. Representative images of 14 replicates per genotype and stained solution are shown. Bars = 200 μm.

2001; Schmittgen and Livak, 2008). For each sample, analyzed in four technical replications, a ΔC_T value was obtained by subtracting the Ubiquitin C_T value from the C_T obtained for the gene of interest. Each data point corresponds to three true biological replicate samples.

Statistical analyses

When appropriate, data were subjected to analyses of variance (ANOVA) and means were compared by the Tukey HSD (Honestly Significant Differences) test ($P \leq 0.05$) using the SPSS Base 12.0 for Windows (SPSS Inc., USA). The Levene's test (for homogeneity of variance) was used prior to ANOVA. Pearson's correlation analyses were carried out using two significance levels ($P \leq 0.05$ and 0.01).

Results and discussion

Fe distribution in grains of different rice cultivars

Through the use of histochemical methods, no Fe in either the ferrous or ferric form was detected in endosperm of the evaluated genotypes. Ferrous Fe was observed in the embryo of only two genotypes: IR75862 and IR69428 (Fig. 1c and d) and was not detected in the aleurone and subaleurone layer. Fe in the ferric form was seen in all genotypes (Fig. 1a and b). Low amounts of ferric Fe were detected in the aleurone layer. For the genotypes which seem to have Fe in the aleurone layer in Fig. 1b images, further analysis of the sections shown in Fig. 1a indicate that the blue color is in fact the reaction present in the subaleurone layer, seen visibly through the aleurone layer. Ferric Fe was visible in the subaleurone layer of BR-IRGA421, BR-IRGA409, IR68144_1, IR75862 and IR69428 (Fig. 1a and b). The subaleurone layer consists of the endosperm's two outermost cell layers, which are known to be rich in proteins and lipids and to have smaller amyloplasts and compound starch granules than the inner endosperm (Juliano, 1993). It is possible to see the reaction in the subaleurone layers, in the external view, due to the fact that the aleurone is generally formed by one layer of cells (Sabelli and Larkins, 2009).

Ferric Fe could be seen in the embryos of all genotypes (Fig. 1a and b). However, the responses to the reaction varied between genotypes, probably due to different Fe content in the tissue. IR69428 was one of the genotypes with the strongest reaction to the potassium ferrocyanide solution, indicating that this genotype's grain has one of the highest Fe concentrations among those evaluated.

Ferric iron, the prevalent Fe form in the rice grain according to our results, could be present in phytin granules, where phytin salts of potassium and magnesium also contain other mineral cations such as Fe^{3+} (Inoue et al., 2009 and references within). Ferric Fe could also be stored in the central cavity of the ferritin protein. However, rice ferritins are probably more involved with oxidative stress protection than with iron storage in the grain (Stein et al., 2009b), similar to *Arabidopsis*, where no more than 5% of total seed Fe is stored in ferritin (Ravet et al., 2009). It was also recently demonstrated that iron and ferritin accumulate in separate cellular locations in *Phaseolus* seeds (Cvitanich et al., 2010).

It would be possible to perform semi-quantitative analysis of Fe using the images obtained (Choi et al., 2007). However, we decided to use a more precise technique, ICP-OES. Moreover, this technique allowed us to evaluate the concentration of Zn, another important micronutrient. Considering that we planned to perform correlation analyses between metal concentrations and gene expression, two more genotypes (IR68144 and IR75862_1) were included in the subsequent analyses.

Fe and Zn concentrations in seeds

Elemental analyses were performed on mature seeds (R9 stage) of eight rice cultivars by ICP-OES (inductively coupled plasma optical emission spectroscopy). Canastra plants were found to have a significantly lower seed Fe concentration ($P \leq 0.05$) relative to all the other cultivars (Table 1), being approximately 45% lower than the highest seed Fe concentrations found in the IR75862, IR68144_1 and IR69428 cultivars. These results corroborate our histochemical data, where IR75862 and IR69428 cultivars showed the presence of ferrous Fe in the embryo (Fig. 1c and d) and IR75862, IR68144_1 and IR69428 cultivars indicated stronger staining of ferric Fe in the subaleurone layer (Fig. 1a and b). Seed Zn concentration was higher in IR75862_1, IR75862, IR68144_1 and IR69428 than in the other four cultivars (Table 1). In order to find the relationship between Fe and Zn concentration in seeds, Pearson's correlation analyses were performed and indicated a positive correlation (0.620, $P = 0.0035$), suggesting that higher Fe and Zn concentrations may occur simultaneously in rice. Positive correlation between seed Fe and Zn concentrations was previously reported in wheat (Cakmak et al., 2004; Morgounov et al., 2007) and rice (Jiang et al., 2007; Sperotto

Table 1
Fe and Zn concentrations in whole seeds from eight diverse rice cultivars. Seeds were collected at full maturity (R9 stage). Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). DW = dry weight.

	Fe (μg g^{-1} DW)	Zn (μg g^{-1} DW)
Canastra	9.83 ± 0.14 c	22.36 ± 0.62 b
BR-IRGA421	12.47 ± 0.24 b	23.59 ± 0.48 b
IR68144	15.11 ± 1.30 ab	21.91 ± 1.05 b
BR-IRGA409	15.22 ± 1.13 ab	22.13 ± 0.25 b
IR75862_1	16.53 ± 0.43 a	34.84 ± 0.75 a
IR75862	17.27 ± 1.25 a	31.07 ± 0.53 a
IR68144_1	17.65 ± 0.60 a	30.73 ± 0.30 a
IR69428	18.74 ± 0.17 a	28.66 ± 0.51 a

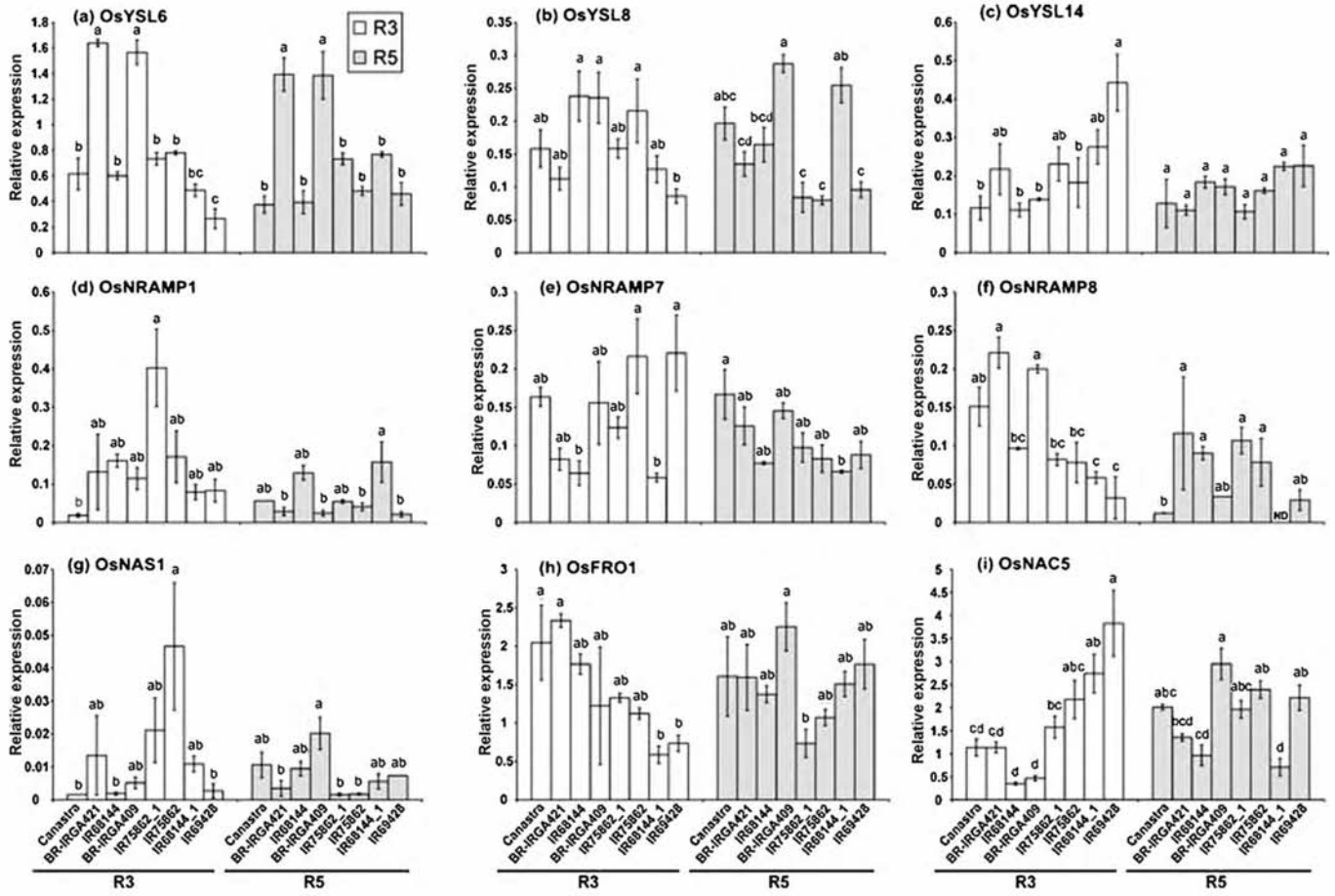

Fig. 2. Relative expression levels (qRT-PCR, relative to Ubiquitin expression) of (a) *OsYSL6*, (b) *OsYSL8*, (c) *OsYSL14*, (d) *OsNRAMP1*, (e) *OsNRAMP7*, (f) *OsNRAMP8*, (g) *OsNAS1*, (h) *OsFRO1* and (i) *OsNAC5* genes in flag leaves of eight diverse rice genotypes collected in R3 (panicle emergence) and R5 (grain filling) stages. Values are the averages of three samples ± SE. Means indicated by different letters are different by the Tukey HSD test ($P \leq 0.05$). ND = not detected. Relative expression levels of tested genes with no statistically significant correlation with Fe and Zn concentrations in whole seeds are shown in Supplementary Figs. 1–4.

et al., 2009). The variation among genotypes was higher in seed Fe concentrations than in Zn concentrations (Table 1). Higher variations in Fe than in Zn concentrations were previously reported (Rengel et al., 1999), including a survey in which Fe and Zn concentrations among genotypes varied 13-fold and 1.2-fold, respectively (Marr et al., 1995).

Gene expression analyses in flag leaves

The expression patterns of 25 Fe and/or Zn-homeostasis related genes (nine genes from the YSL family of metal-phytosiderophore transporters: *OsYSL2*, *OsYSL5*, *OsYSL6*, *OsYSL7*, *OsYSL8*, *OsYSL10*, *OsYSL14*, *OsYSL15* and *OsYSL18*; six genes from the NRAMP family of metal transporters: *OsNRAMP1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsNRAMP7* and *OsNRAMP8*; four genes from the ZIP family of divalent metal transporters: *OsZIP4*, *OsZIP5*, *OsZIP6* and *OsZIP7*; two genes from the NAS family of enzymes necessary for nicotianamine biosynthesis: *OsNAS1* and *OsNAS2*; one gene from the IRT family of Fe^{2+} transporters: *OsIRT1*; one gene of the FRO family of ferric-chelate reductase/oxidase protein: *OsFRO1*; one gene of the vacuolar iron transporter family: *OsVIT1* and one gene from the NAC family of transcription factors: *OsNAC5*) were analyzed in flag leaves of eight different rice cultivars (showing contrasting levels of Fe and Zn in the seeds) during two reproductive developmental stages: R3 (panicle emergence) and R5 (grain filling) (Fig. 2 and Supplementary Figs. 1–4). The locus ID numbers of the 25 analyzed genes, according to the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), are presented in Supplementary Table 2.

The expression pattern of the tested genes varied among the eight cultivars. Surprisingly, there were no genes with up-regulated expression in R5 flag leaves relative to R3 across all tested cultivars. When differences were seen, they usually occurred only in one to a few cultivars, and were not a consistent trend. Most of the genes were found to be expressed in flag leaves, and expression of *OsYSL6*, *OsFRO1* and *OsNAC5* genes reached higher levels than *OsUBQ* expression (Fig. 2). Other genes (*OsNAS1*, *OsNAS2*, *OsNRAMP4*, *OsNRAMP5*, *OsZIP4*, *OsZIP5* and *OsIRT1*) exhibited low to non-detectable expression in flag leaves (Fig. 2 and Supplementary Figs. 2–4). Expression of *OsNAC5*, *OsYSL10*, *OsYSL14*, *OsYSL15*, *OsYSL18*, *OsNRAMP5*, *OsZIP5*, *OsIRT1* and *OsVIT1* genes was high at the R3 stage of at least one of the highest Fe and Zn cultivars (IR75862 _1, IR75862, IR68144_1 and IR69428) (Fig. 2 and Supplementary Figs. 1–4). On the other hand, expression of *OsYSL6*, *OsYSL7*, *OsYSL8* and *OsNRAMP8* genes was low at the R3 stage in the highest Fe cultivar (IR69428) (Fig. 2 and Supplementary Fig. 1). There were no major differences in gene expression during the R5 stage among the eight tested cultivars.

Correlation between gene expression in flag leaves and Fe and Zn concentration in seeds

To search for relationships between gene expression in flag leaves during different stages of reproductive development (R3 and

Table 2
Correlation analyses between expression data of nine metal-related genes in flag leaves during the reproductive development (stages R3 and R5) and Fe and Zn concentrations in seeds from eight rice cultivars (Canastra, BR-IRGA421, BR-IRGA409, IR68144, IR75862_1, IR68144_1, IR75862 and IR69428). A complete list of all genes tested is shown in Supplementary Table 3.

Gene	Fe		Zn	
	R3	R5	R3	R5
OsYSL6	−0.322	−0.116	−0.400*	−0.209
OsYSL8	−0.109	−0.205	−0.307	−0.462*
OsYSL14	0.539**	−0.150	0.406*	−0.097
OsNRAMP1	0.244	0.152	0.518**	0.061
OsNRAMP7	0.084	−0.602**	0.139	−0.416*
OsNRAMP8	−0.606**	0.053	−0.605**	0.224
OsNAS1	0.199	−0.182	0.471*	−0.535**
OsFRO1	−0.709**	−0.109	−0.510**	−0.359
OsNAC5	0.512*	−0.035	0.528**	−0.064

* Significant at 0.05 probability level.
** Significant at 0.01 probability level.

R5) and final Fe and Zn concentrations in seeds, Pearson's correlation analyses were performed for eight different rice cultivars. Final seed Fe concentration was positively correlated with *OsYSL14* and *OsNAC5* expression in flag leaves during R3 and negatively correlated with *OsNRAMP8* and *OsFRO1* expression during R3 and with *OsNRAMP7* expression during R5 (Table 2). Final seed Zn concentration was positively correlated with *OsYSL14*, *OsNRAMP1*, *OsNAS1* and *OsNAC5* expression in flag leaves during R3 and negatively correlated with *OsYSL6*, *OsNRAMP8* and *OsFRO1* expression during R3 and with *OsYSL8*, *OsNRAMP7* and *OsNAS1* expression during R5 (Table 2).

Fig. 2 shows the expression profile of the genes for which we found significant correlations between expression in flag leaves during either R3 or R5 and final seed Fe or Zn concentration. The bars representing the different cultivars are shown (from left to right) according to the ascending order of final Fe concentrations in the same cultivars. Some correlations can be clearly visualized, such as the negative correlations of *OsNRAMP8* (Fig. 2f) and *OsFRO1* (Fig. 2h) and the positive correlations of *OsYSL14* (Fig. 2c) and *OsNAC5* (Fig. 2i) expression with Fe during R3.

These analyses allowed us to assess which of these genes might contribute positively or negatively to the process of Fe or Zn transport from flag leaves to the seeds. However, we acknowledge that this process is very complex and that the 25 genes analyzed in our study represent only a small set of the molecular players that may help mobilize metals from flag leaves (and possibly non-flag leaves) to developing seeds.

The YSL family of transporters is believed to transport NA–metal chelates across plant cell membranes. Experimental evidence points to a role of the YSL proteins in the long-distance and intracellular transport of metals, especially Fe, complexed to NA (Curie et al., 2009; Ishimaru et al., 2010). Of the nine tested genes from the YSL family of metal–NA transporters, the expression levels of two (*OsYSL6* and *OsYSL8*) showed negative correlation with final Zn concentration in the seeds and one (*OsYSL14*) showed positive correlation with final Fe and Zn concentrations in the seeds (Table 2). None of these transporters has been functionally characterized. The *OsYSL14* gene product was previously suggested to be involved in the movement of metals within the plant, since the expression pattern of this gene is restricted to aerial plant organs (Nishizawa, 2006). The negative correlation found for *OsYSL6* and *OsYSL8* could be explained by the putative role of YSL proteins in metal movement to the vacuole. In this way, higher expression of such genes could result in higher proportion of Fe and Zn unavailable to be transported to seeds. Interestingly, AtYSL4 and AtYSL6 were identified in an *Arabidopsis* tonoplastic proteomic study (Jaquinod et al., 2007), and according to Curie et al. (2009), *AtYSL4* and *AtYSL6*, together with the two rice members *OsYSL5* and *OsYSL6*, form a separate cluster in the YSL family, suggesting that both rice proteins could also be located to the tonoplast and cause metal–NA complexes to flow out from the cytoplasm to the vacuolar compartment (Curie et al., 2009). If localized in the plasma membrane, the OsYSL5 and OsYSL6 proteins could be necessary for metal uptake into leaf cells with high metal demand, also decreasing metal availability for phloem loading.

In addition to the YSL genes, the expression level of the enzyme-coding gene *OsNAS1* in flag leaves during the R3 stage was positively correlated with and during the R5 stage was negatively correlated with Zn concentration in the seeds. NAS is required for the biosynthesis of NA, a non-peptidyl metal chelator that is believed to be a co-substrate of the YSL proteins (Roberts et al., 2004; Schaaf et al., 2004). Fe and Zn are potentially chelated by NA during phloem transport (von Wirén et al., 1999) and NAS genes are expressed in cells involved in long-distance transport of Fe (Inoue et al., 2003). Overexpression of barley *NAS* gene in tobacco increased Fe and Zn concentrations in young leaves, flowers and seeds of transgenic plants (Takahashi et al., 2003), indicating that NA promoted the transport of Fe and Zn to young leaves and reproductive organs in dicotyledonous plants.

The family of natural resistance-associated macrophage protein (NRAMP) metal ion transporters plays a major role in metal ion homeostasis in different species from bacteria to human (Nevo and Nelson, 2006). Of the six tested genes from the NRAMP family, one (*OsNRAMP1*) showed positive correlation with final Zn concentration in the seeds and two (*OsNRAMP7* and *OsNRAMP8*) showed negative correlation with final Fe and Zn concentrations in the seeds (Table 2). Several NRAMP proteins have been shown to transport multiple classes of cations, suggesting that NRAMP proteins may exhibit broad substrate specificity (Curie et al., 2000). OsNRAMP1 belongs to the same class as AtNRAMP1, which was shown to transport Fe (Curie et al., 2000). The subcellular localization of AtNRAMP3 on the vacuolar membrane suggests a function in intracellular metal homeostasis (Thomine et al., 2003). *OsNRAMP7* is the closest rice homolog to *AtNRAMP3* (data not shown). According to Stangoulis et al. (2007), there is a QTL for grain Fe concentration on chromosome 12 explaining approximately 14% of the phenotypic variation. Co-located with this QTL for Fe, there is a QTL for grain Zn concentration explaining approximately 13% of the phenotypic variation (Stangoulis et al., 2007; Garcia-Oliveira et al., 2009). A more in depth analysis revealed that *OsNRAMP7* locates inside this QTL (data not shown). However, it is still unknown whether NRAMP proteins drive metal influx or efflux from the vacuole. Based on our correlation analysis, OsNRAMP1 could function as a metal efflux transporter (participating in the export of metals from the vacuolar compartment to the cytosol), resulting in increased metal concentration available to be transported to the seeds. OsNRAMP7 and 8 could function as metal influx proteins (participating in the vacuolar sequestration of metals). However, the demonstration of this hypothesis requires further investigation.

In Strategy I plants, the reduction of Fe^{3+} is a prerequisite for the transport of Fe^{2+}, and Fe-deficiency leads to activation of Fe^{3+}-chelate reductase at the root surface. However, in rice, no Fe^{3+} reductase activity is detected at the root surface (Ishimaru et al., 2006). In leaves, *OsFRO1* is expressed under Zn-, Mn- and Cu-deficiency (Ishimaru et al., 2006). However, we were able to detect high expression of *OsFRO1* in flag leaves of rice plants grown under usual field conditions (Fig. 2), indicating that reduction steps may be needed for internal mineral transport. Our data show that *OsFRO1* expression in R3 flag leaves negatively correlates with Fe and Zn concentrations in seeds. According to Jeong et al. (2008), AtFRO7 plays a role in chloroplast Fe acquisition. Phylogenetic analyses show that *OsFRO1* and *AtFRO7* are closely related (Vinícius de Abreu Waldow, personal communication). In this way, OsFRO1

could have similar function in rice, directing Fe to the chloroplasts and avoiding its mobilization from flag leaves. A possible role for OsFRO1 in import of Fe to chloroplasts would not be unexpected given the importance of Fe for photosynthesis. In addition, it is thought that Fe storage in plants mainly occurs in plastids as the Fe storage protein, ferritin, is plastid-localized in plants (Zancani et al., 2007). It is already known that Fe^{3+} reduction is necessary for the subsequent uptake by ferritin (Laulhere and Briat, 1993). However, Fe^{3+} reduction is probably needed in a variety of other locations.

OsNAC5 has been recently characterized as a novel senescence-associated ABA-dependent NAC transcription factor (Sperotto et al., 2009). Higher and earlier *OsNAC5* expression is seen in flag leaves and panicles of IR75862 plants, which have higher seed Fe, Zn and protein concentrations than other tested genotypes (Sperotto et al., 2009). We found a positive correlation between the expression of this gene during the panicle emergence stage (R3) in flag leaves and final Fe and Zn concentrations in the grains. Lu et al. (2008) identified in rice a QTL (*qZn-11*) for grain Zn concentration on chromosome 11, between markers C794 and RG118, accounting for 18.61% of the phenotypic variation. A detailed analysis revealed that *OsNAC5* locates inside this QTL (data not shown). In wheat, the presence of the Gpc-B1 allele confers earlier flag leaf senescence and it was suggested that the Gpc-B1 locus is involved in more efficient remobilization of amino acids, Zn, Fe and Mn from leaves to grains, in addition to its effect on earlier senescence of the green tissues (Uauy et al., 2006). These effects are caused by the *NAM-B1* gene, located in the Gpc-B1 locus, which encodes a NAC domain-containing protein (Uauy et al., 2006; Waters et al., 2009). It is already known that a large number of metal transporter proteins are up-regulated during *Arabidopsis* leaf senescence (Van der Graaff et al., 2006). It is possible that OsNAC5 protein regulates similar transporter genes in rice and that these genes are needed for effective Fe and Zn remobilization.

Surprisingly, most of the correlations found between metal-related genes expression in flag leaves with final seed Fe and Zn concentrations (Table 2) were found during R3 (panicle emergence stage), instead of during R5 (grain filling stage). Mineral remobilization in early stages of reproductive growth has been demonstrated in wheat. Expression of *NAM-B1* is responsible for the earlier onset of flag leaf senescence, resulting in more efficient remobilization of protein, Zn, Fe and Mn from leaves to the grains (Uauy et al., 2006). *NAM-B1* expression is seen right after anthesis (Uauy et al., 2006), which in rice corresponds to R4 stage (Counce et al., 2000). Moreover, the highest seed Zn concentration following foliar Zn applications in wheat was found at the beginning of seed development (around 10 days after anthesis), suggesting that applying foliar Zn during early stages of wheat reproductive development could be an effective way of increasing seed Zn concentration through efficient flag leaf export to the grain (Ozturk et al., 2006). More studies are needed to evaluate if this critical period for increasing seed metal concentration in rice occurs not only after anthesis but also during the R3 stage.

It is possible that other mechanisms of mineral allocation to the rice grain, such as remobilization from other leaves, phloem delivery of minerals acquired from the xylem through a transfer process or even direct xylem loading, may contribute more Fe and Zn than that of flag leaf remobilization during grain filling in rice. However, flag leaf remobilization is one mechanism that could be enhanced in biofortification efforts, provided that the relevant genes and gene products are identified and their contributions to the process understood.

The lack of statistical correlation between gene expression in the flag leaves and metal concentration in the seeds does not mean that such gene/gene products have no function in the process of Fe and/or Zn transport to distant sinks, such as seeds. A study with a higher number of cultivars and/or with a different set of genes could reveal other significant correlations. It is also important to consider that changes in mRNA levels may not correlate with changes in protein or enzyme activity, due to post-transcriptional processes. Nevertheless, the expression profiles defined in this study do provide a useful starting point for more in depth analyses of the molecular mechanisms behind Fe and Zn remobilization from flag leaves to rice seeds. Functional analyses of selected gene products, using yeast complementation studies to verify their transport capacities should be done to confirm their involvement with this process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2010.05.003.

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Supplementary Table 1 Gene-specific PCR primers used for qRT-PCR.

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'	Amplicon size (bp)
OsYSL2	CTCCTCCTCCTCCCGGC	TCTTCCTGTTCAGAGCCAGC	131
OsYSL5	CATGTCCTTCAGGCCATCTT	GGACAACGGCTCTATTCCAA	192
OsYSL6	TGTGCATGTACTTCAAGCCATC	AAGAACAAAGTTACTGCACTTTTGC	150
OsYSL7	GGGAAACTAGATCATTTTCTCAGG	GGTAGAGGAGATCAAACTGTGTTTA	139
OsYSL8	CTCAAGCTAGCCTTCCATCG	TGCTACACCAGCTGCTTCTC	150
OsYSL10	CCCCTCCTCCTAGCTACCAT	CATGCGGGCTACTAGCAAAG	182
OsYSL14	CCGGTTAGTCGTGCCATC	ATCTGGAAATACATTTGGAGGAGA	100
OsYSL15	GGATTGCAGAAATAAACAGTGATG	TGCCAAACTAAACAATTCTCAA	167
OsYSL18	TCTTGATCGAGGAAGAAGTGG	TGCCATAGTATGTTCGTTGGA	162
OsNRAMP1	CGGTGTTGGCTGGTTTTTAT	CATTCTGCCAATCTGCCAAT	193
OsNRAMP4	TTGCTTGCTGAGTAGTGCAT	GCTGCTTAGAAACAACAACAAGAA	126
OsNRAMP5	GTCGGAGCCGTTCGTTTAT	GGCTCTGCCCTGAATTATGA	159
OsNRAMP6	GCTCAAAGCCTCGAAATCAT	TGGCGTGGAAGAGAATTTTA	141
OsNRAMP7	GCTGCCAAATCAGATCATCA	GCTTCAGGACGACACAGTCA	240
OsNRAMP8	GTTTGGGGATGACCATTTTG	GTGCCTTTGCTCCATTCTGT	223
OsZIP4	GATTCTTGGGCAAATGGTGT	ACAACGCTGGGGATTATTTG	258
OsZIP5	CAGGAATGGCAGGTTTTTGT	AGTTTCAACCAACGGAGTGG	113
OsZIP6	GCAACCAGAGTGAAATACGG	AGGAGACGAAAATGGCTCA	154
OsZIP7	TGCACAACAACGGATACAGA	TCAGCCAACAACACTCTCCA	251
OsNAS1	GTCTAACAGCCGGACGATCGAAAGG	TTTCTCACTGTCATACACAGATGGC	264
OsNAS2	CAGACGGTCACAAACACCTCTTGC	TGAGTGCGTGCATAGTAATCCTGGC	234
OsIRT1	GCAATTCGCTGCATTGTTAG	GAAGTACATCATCAGTCACGAA	162
OsFRO1	CTTCTTCTACCCGACCAAGC	GATATGGTCACTCGGGAAGG	172
OsVIT1	GTGCCACTCCTACCCTACA	TAAACGGGCCCTTGACATAG	112
OsNAC5	CAGCAGCTGATGGTATTGTC	AGAGACCTGTTTGGCACGAA	109
OsUBQ	AACCAGCTGAGGCCCAAGA	ACGATTGATTTAACCAGTCCATGA	77

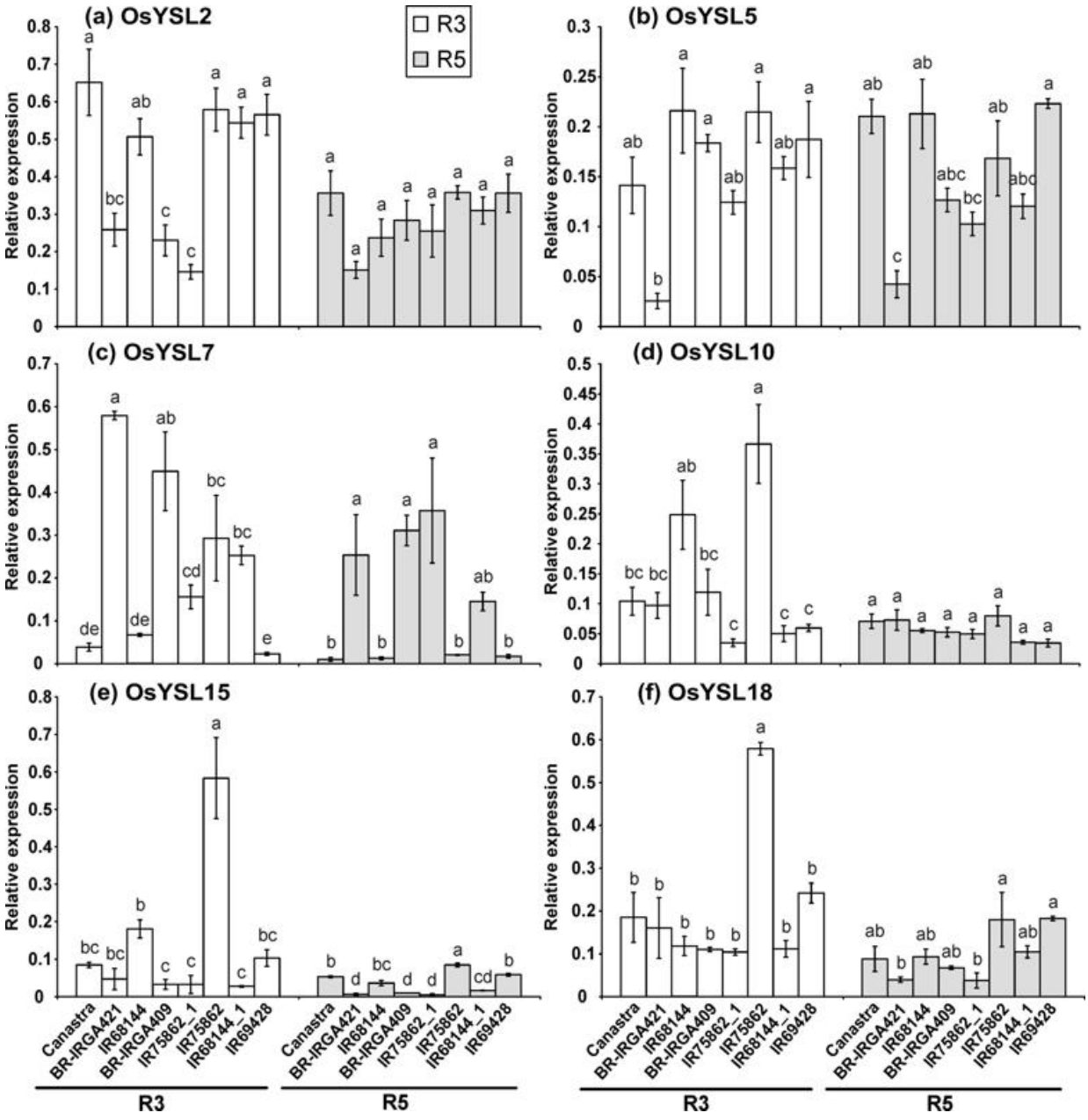
Supplementary Table 2 Locus ID numbers of the 25 analyzed genes.

	Gene	Locus ID number
YSL family of metal-phytosiderophore transporters	*OsYSL2*	Os02g43370
	OsYSL5	Os04g32060
	OsYSL6	Os04g32050
	OsYSL7	Os02g02450
	OsYSL8	Os02g02460
	OsYSL10	Os04g57840
	OsYSL14	Os02g42220
	OsYSL15	Os02g43410
	OsYSL18	Os01g61390
NRAMP family of metal transporters	*OsNRAMP1*	Os07g15460
	OsNRAMP4	Os02g03900
	OsNRAMP5	Os07g15370
	OsNRAMP6	Os01g31870
	OsNRAMP7	Os12g39180
	OsNRAMP8	Os03g41070
ZIP family of divalent metal transporters	*OsZIP4*	Os08g10630
	OsZIP5	Os05g39560
	OsZIP6	Os05g07210
	OsZIP7	Os05g10940
NAS family of nicotianamine biosynthesis	*OsNAS1*	Os03g19427
	OsNAS2	Os03g19420
IRT family of Fe^{2+} transporters	*OsIRT1*	Os03g46470
FRO family of ferric-chelate reductase/oxidase	*OsFRO1*	Os04g36720
Vacuolar iron transporter family	*OsVIT1*	Os09g23300
NAC family of transcription factors	*OsNAC5*	Os11g08210

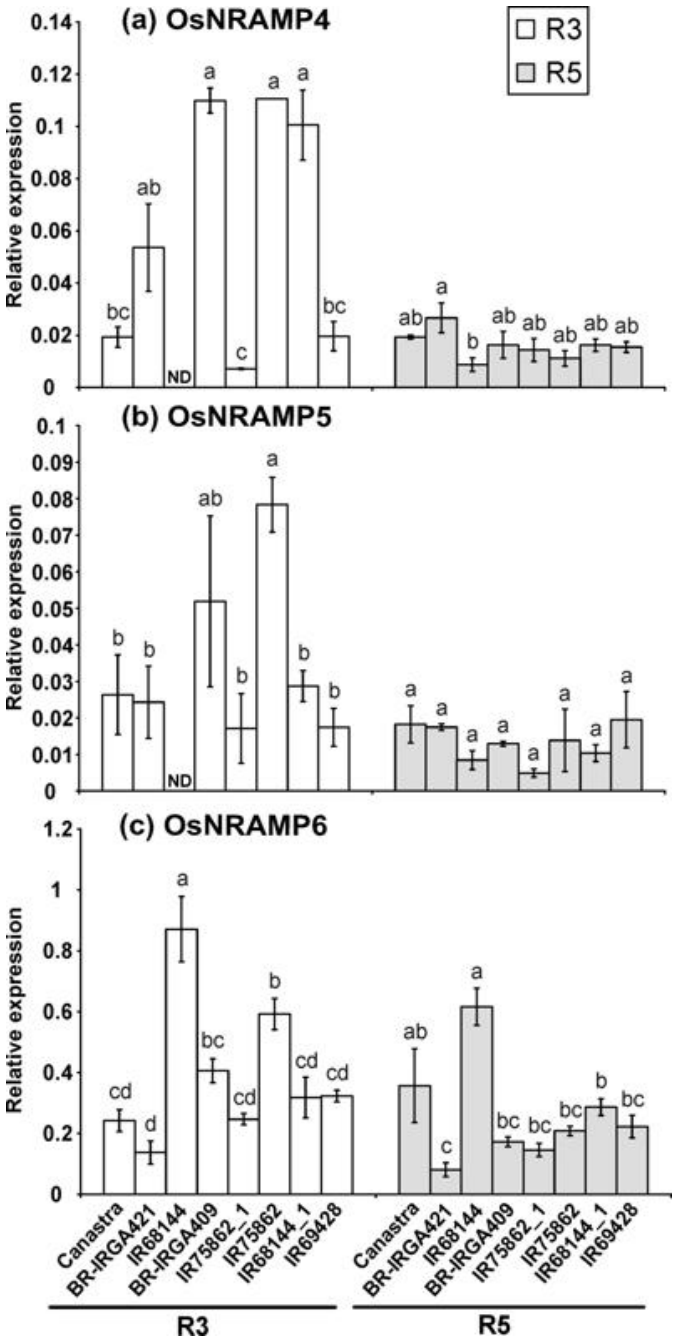
Supplementary Table 3 Correlation analyses between expression data of 25 metal-related genes in flag leaves during the reproductive development (stages R3 and R5) and Fe and Zn concentrations in seeds from eight rice cultivars (Canastra, BR-IRGA421, BR-IRGA409, IR68144, IR75862_1, IR68144_1, IR75862 and IR69428).

Gene	Fe		Zn	
	R3	R5	R3	R5
OsYSL2	-0.100	0.058	-0.140	0.189
OsYSL5	0.392	0.006	-0.044	-0.221
OsYSL6	-0.322	-0.116	**-0.400***	-0.209
OsYSL7	-0.042	0.015	-0.142	0.101
OsYSL8	-0.109	-0.205	-0.307	**-0.462***
OsYSL10	0.010	-0.349	-0.078	-0.174
OsYSL14	**0.539****	-0.150	**0.406***	-0.097
OsYSL15	0.252	0.009	0.299	0.074
OsYSL18	0.220	0.382	0.274	0.233
OsNRAMP1	0.244	0.152	**0.518****	0.061
OsNRAMP4	0.278	-0.318	0.004	-0.162
OsNRAMP5	0.039	-0.395	-0.093	-0.252
OsNRAMP6	0.258	-0.141	-0.213	-0.329
OsNRAMP7	0.084	**-0.602****	0.139	**-0.416***
OsNRAMP8	**-0.606****	0.053	**-0.605****	0.224
OsZIP4	-0.141	-0.154	-0.041	-0.285
OsZIP5	0.096	0.199	-0.096	0.075
OsZIP6	-0.064	-0.378	-0.060	-0.173
OsZIP7	0.072	-0.428	0.078	-0.334
OsNAS1	0.199	-0.182	**0.471***	**-0.535****
OsNAS2	-0.062	0.067	-0.164	-0.088
OsIRT1	0.087	0.013	-0.084	-0.142
OsFRO1	**-0.709****	-0.109	**-0.510****	-0.359
OsVIT1	-0.131	-0.081	0.061	0.097
OsNAC5	**0.512***	-0.035	**0.528****	-0.064

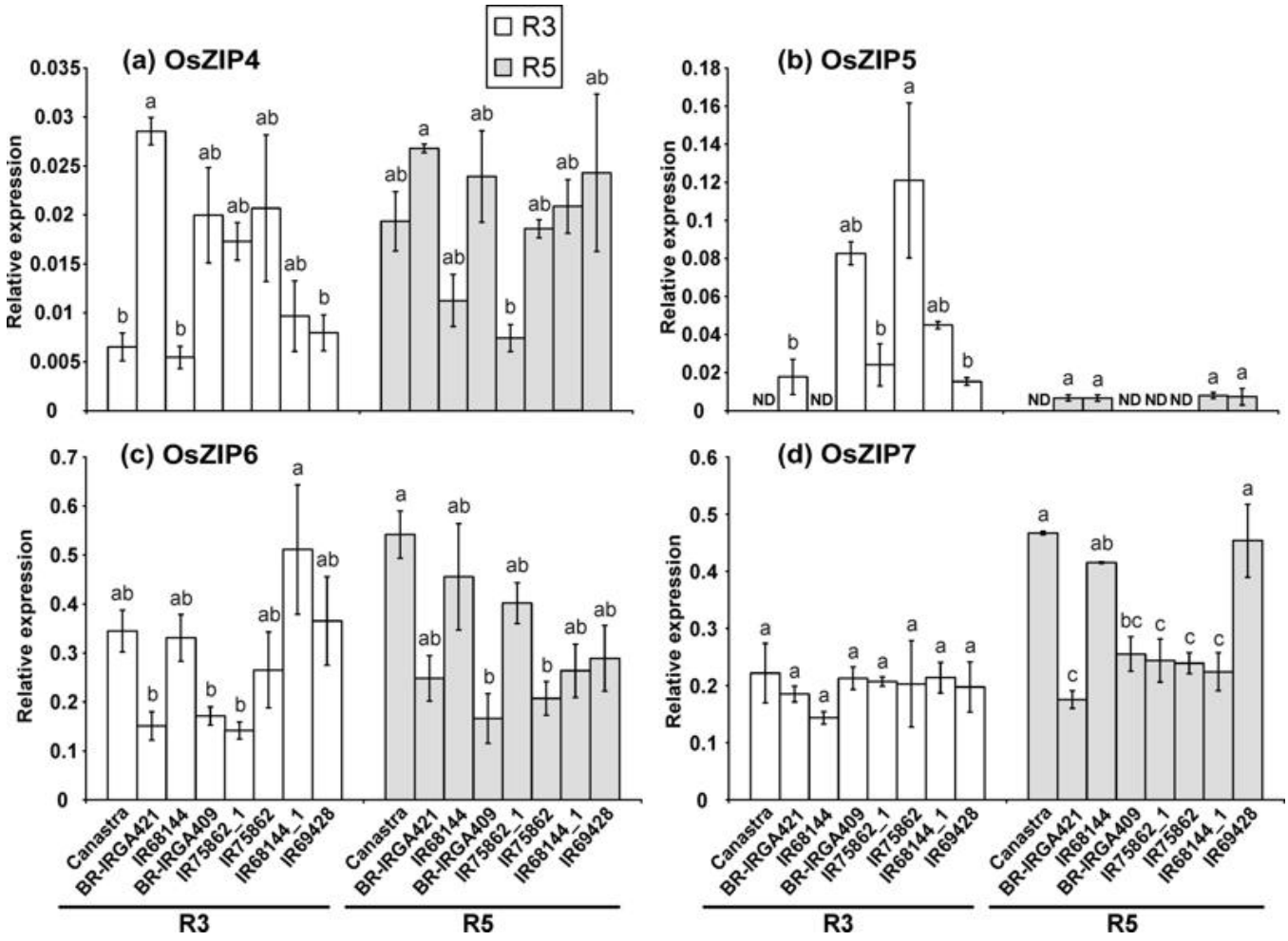
Supplementary Figure 1



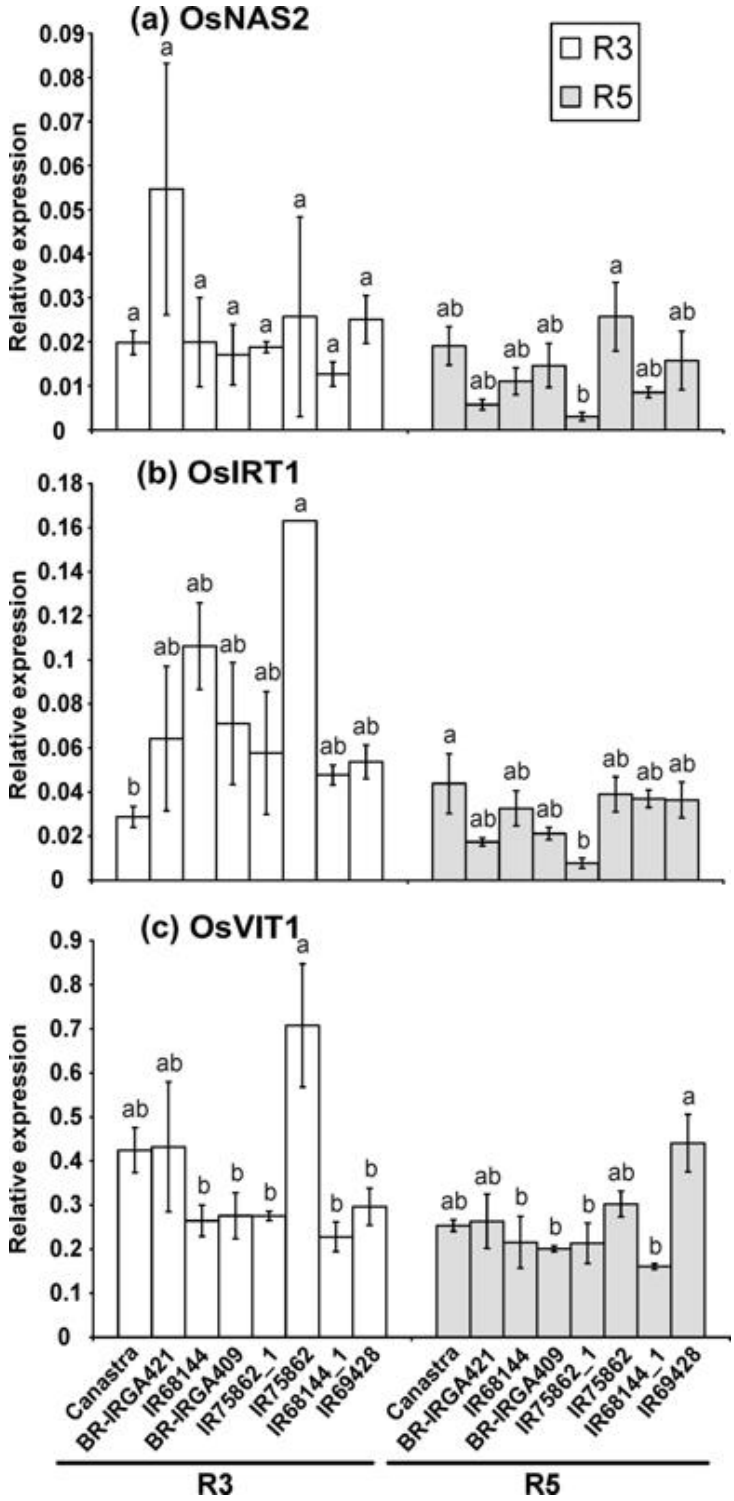
Supplementary Figure 2



Supplmentary Figure 3



Supplementary Figure 4



Discussão Geral

As folhas-bandeira desempenham um papel essencial na mobilização de fotoassimilados via floema para os grãos de arroz, mas ainda não é sabido se as folhas-bandeira desempenham papel semelhante na remobilização de metais para os grãos. Sabe-se que outros processos contribuem (talvez mais ativamente que a mobilização a partir das folhas-bandeira) para o acúmulo de metais nos grãos de arroz, como mobilização a partir de outras partes da planta, contínua absorção pelas raízes durante o enchimento do grão e transporte direto via xilema. Entretanto, ao menos uma pequena parte do Fe e Zn encontrados nos grãos deve ser proveniente das folhas-bandeira. Logo, a identificação de genes importantes nesse processo pode fornecer alvos para futuros programas de melhoramento tradicional ou transgenia, visando o aumento nas concentrações de Fe e Zn nos grãos de arroz.

Concentração de Fe, Zn e proteínas nos grãos de arroz

Através da quantificação de metais e proteínas em grãos maduros de diferentes cultivares de arroz, foi identificada correlação positiva entre as concentrações de Fe, Zn e proteínas. Outros metais não apresentaram correlação significativa com as concentrações de proteínas. Correlação positiva entre Fe e Zn já foi mostrada em trigo (Cakmak et al. 2004; Morgounov et al. 2007) e arroz (Jiang et al. 2007a). Os conteúdos de Mg, Zn, Cu e Mn apresentaram correlação positiva com o conteúdo de proteínas em sementes de arroz (Jiang et al. 2007a) e em trigo foi demonstrada uma clara associação entre as concentrações de Fe, Zn, Mn e proteínas nos grãos (Distelfeld et al. 2007).

Nosso trabalho é o primeiro a mostrar uma correlação positiva entre as concentrações de Fe, Zn e proteínas nos grãos de arroz, sugerindo que altas concentrações desses dois metais e proteínas devem ocorrer simultaneamente nestes grãos. Esta ocorrência simultânea pode indicar que esses dois metais sejam transportados para os grãos através dos mesmos mecanismos, uma vez que existem transportadores de Fe que são capazes de transportar Zn (Guerinot, 2000; Schaaf et al. 2005), o que poderia explicar, pelo menos em parte, a alocação simultânea dos dois metais. Entretanto, mecanismos de transporte mais específicos também devem existir no transporte de Fe e Zn. A contribuição relativa de cada um dos mecanismos de transporte

de Fe e Zn (compartilhados e específicos) é desconhecida em arroz. Em plantas de ervilha e feijão, o Fe aplicado nas folhas é transportado até os grãos com maior eficiência que o Zn. Em torno de 40% da dose de Fe aplicada foi encontrada nos grãos, enquanto que apenas 5-8% do Zn foi encontrado, indicando que a regulação do transporte para os grãos é diferente para cada micronutriente (Fawzi et al. 1993; Rengel et al. 1999).

A variação na concentração de Fe entre as cultivares de arroz testadas foi maior que a variação na concentração de Zn. Numa análise de genótipos australianos, foi visto uma variação de mais de 13 vezes na concentração de Fe nos grãos, enquanto que a variação na concentração de Zn foi de apenas 1,2 vezes (Marr et al. 1995). Esta variação maior na concentração de Fe do que na concentração de Zn parece ser normal na natureza, mas a explicação pra essa diferença é desconhecida. Outros estudos mostraram uma grande variação na concentração de Fe nos grãos entre os genótipos de arroz testados (Prom-u-thai et al. 2007; Pintasen et al. 2007; Sellappan et al. 2009).

Construção da biblioteca de SSH e análise dos genes induzidos em folhas-bandeira

Através da análise de duas cultivares por SSH, foi possível isolar 78 seqüências induzidas em folhas-bandeira de arroz durante a fase de enchimento do grão (R5) em relação à fase de emergência da panícula (R3), de acordo com a classificação de Counce et al. (2000). Dentre essas seqüências, foram encontradas onze que codificam proteínas transportadoras (que podem estar envolvidas com o processo de enchimento e mobilização de metais para os grãos de arroz - Takeda et al. 2001; Yamaguchi et al. 2002; Curie e Briat, 2003; Scofield et al. 2007; Stacey et al. 2008; Vasconcelos et al. 2008; Wang et al. 2008), uma que codifica a enzima nicotianamina sintase (que pode estar envolvida com o transporte a longa distância de Fe e Zn, uma vez que cataliza a formação de nicotianamina, o principal quelante de Fe e Zn no floema - von Wirén et al. 1999; Nishizawa, 2005), quatro previamente associadas com processos de senescência (Sperotto et al. 2007; Sperotto et al. 2008 - indicando a ocorrência de um processo inicial de senescência na fase de enchimento do grão, que pode afetar a mobilização de metais das folhas-bandeira) e dez fatores de transcrição (que podem regular o processo de mobilização de metais das folhas-bandeira para os grãos - Waters et al. 2009).

Entre as seqüências que codificam proteínas transportadoras, foram encontradas três (*OsMST1*, *OsMST3* e *OsSTP*) que devem participar do processo de transporte de fotoassimilados das folhas-bandeira para os grãos. Como as folhas-bandeira são a principal fonte de fotoassimilados para os grãos, o aumento na expressão desses genes já era esperado. De fato, já foi demonstrado que genes transportadores de açúcares têm a função de suprir o grão em desenvolvimento (Wang et al. 2008). Caso a capacidade de transporte de açúcares (ao longo do caminho entre a folha-bandeira até a base do grão) desses três possíveis genes transportadores encontrados no SSH seja confirmada, poderiam ser utilizados para a biofortificação de Fe e Zn em grãos de arroz. Para tanto, a região promotora desses genes seria fusionada com região codificadora de outros genes importantes para o processo. Dessa forma, a expressão desse transgene seria direcionada para o caminho entre a folha-bandeira e o grão, favorecendo o transporte de Fe e Zn para os grãos em desenvolvimento. Recentemente, Ishimaru et al. (2010) fusionou o promotor do transportador de sacarose *OsSUT1* com o gene *OsYSL2* e verificou um aumento no acúmulo de Fe no endosperma de arroz, um padrão consistente com a expressão de *OsSUT1*.

A enzima nicotianamina sintase (*OsNAS3*), cuja seqüência codificante foi encontrada no SSH, já foi sugerida por Nishizawa (2005) como um alvo para aumentar a concentração de metais em grãos de arroz. Entretanto, plantas contendo mutações nesse gene ainda não foram analisadas, o que seria de extrema importância para a elucidação da real função desta enzima.

Entre as seqüências previamente associadas com processos de senescência, encontramos uma proteína cuja função é desconhecida (J023008A06 *Senescence-associated protein*). Mais análises deveriam ser feitas para verificar se a expressão desse gene é induzida somente após o estabelecimento de um processo de senescência ou se é importante no estabelecimento da senescência foliar em arroz.

Entre os fatores de transcrição, foi encontrado *OsNAC5*. A família de proteínas NAC compreende um grande número de proteínas de plantas identificáveis pela presença de um domínio NAC altamente conservado na posição N-terminal. As proteínas NAC já foram descritas como envolvidas no controle transcricional de um grande número de processos em plantas, como desenvolvimento meristemático, formação de raízes laterais, sinalização por auxina, defesa e resposta a estresse abiótico (Olsen et al. 2005). De acordo com Fang et al. (2008), *OsNAC5* e *6* pertencem a subfamília SNAC (*stress-responsive NAC*). A expressão de *OsNAC6* é induzida por

estresses abióticos como frio, seca, alta salinidade e aplicação de ácido abscísico (ABA) (Ohnishi et al. 2005), além de estresses bióticos (Nakashima et al. 2007). A expressão de genes da família NAC em folhas senescentes já foi demonstrada por diversos grupos (Guo et al. 2004; Lin e Wu, 2004; Buchanan-Wollaston et al. 2005; Guo e Gan, 2006). *AtNAP*, que codifica um fator de transcrição da família NAC, está associado ao processo de senescência foliar em *Arabidopsis*, e o fenótipo mutante pode ser restaurado pelo seu ortólogo em arroz (*OsNAP* ou *ONAC058* - Guo e Gan, 2006). O gene *ONAC058* também pertence à subfamília SNAC de arroz (Fang et al. 2008). O padrão de expressão associado à senescência de outros 20 membros da família NAC sugere que a família NAC tem um papel importante na senescência foliar (Guo et al. 2004; Buchanan-Wollaston et al. 2005). *Gpc-B1*, um QTL de trigo, está envolvido no aumento da eficiência de remobilização de aminoácidos, Zn, Fe e Mn das folhas para os grãos (Uauy et al. 2006a; Distelfeld et al. 2007; Waters et al. 2009). Esse alelo ancestral de trigo codifica um fator de transcrição NAC (NAM-B1), que regula a senescência e aumenta a remobilização de nutrientes. A redução nos níveis de mRNA de múltiplos homólogos de *NAM-B1* através de RNAi atrasou a senescência em mais de três semanas e reduziu em mais de 30% o conteúdo de proteínas, Zn e Fe nos grãos de trigo (Uauy et al. 2006b). Análises filogenéticas revelaram que a proteína de arroz mais próxima da proteína NAM-B1 de trigo é ONAC010 (Os07g37920) (Uauy et al. 2006b). Este gene não corresponde ao gene *OsNAC5* encontrado em nossos experimentos (Os11g08210). Uma vez que fatores de transcrição NAC foram descritos como associados a processos de senescência (Guo et al. 2004; Buchanan-Wollaston et al. 2005) e remobilização de nutrientes (Uauy et al. 2006b), decidimos caracterizar o gene *OsNAC5*.

Análise do domínio NAC

As seqüências de aminoácidos do domínio NAC das proteínas OsNAC5 e ONAC010 apresentam 65 e 86% de identidade, respectivamente, com o domínio NAC da proteína NAM-B1 de trigo. De acordo com Fang et al. (2008), a família NAC de arroz pode ser classificada em cinco grupos (I-V), e análises filogenéticas mostraram que *OsNAC5* e *ONAC010* pertencem ao grupo III (*stress-responsive NAC genes* - *SNAC*). Através da composição de motivos encontrados no domínio NAC, a família NAC de arroz pode ser classificada em 15 tipos (A-O) (Fang et al. 2008; Ganesan et al.

2008). OsNAC5 e ONAC010 pertencem ao grupo A e possuem um domínio completo de ligação a DNA (cinco motivos nomeados de a até e) e um domínio de regulação transcricional variável (Fang et al. 2008; Ganesan et al. 2008). Foi constatado que o gene *ONAC010* não é expresso em folhas-bandeira no estágio R3 nem no estágio R5. De acordo com Fang et al. (2008), *ONAC010* é expresso somente nos estames e sua expressão é induzida por seca e alta salinidade. Esses dados sugerem que o ortólogo do NAM-B1 de trigo não desempenha a mesma função em arroz. Dessa forma, é importante analisar outros fatores de transcrição NAC de arroz que podem estar afetando a senescência e o processo de mobilização de nutrientes.

Expressão de *OsNAC5* e concentração de Fe e Zn nas folhas-bandeira

A avaliação da expressão de *OsNAC5* em folhas-bandeira de quatro cultivares com níveis contrastantes de Fe e Zn nos grãos mostrou que esse gene tem maior expressão (além de expressão adiantada) na cultivar que possui os maiores níveis de Fe, Zn e proteína nos grãos (IR75862), sugerindo que uma maior (e anterior) expressão desse gene poderia resultar num maior aporte de Fe, Zn e proteínas para os grãos. Através da análise da concentração de metais em folhas-bandeira, a única cultivar que mostrou um decréscimo na concentração de Fe no estágio R5 (em relação ao estágio R3) foi a cultivar IR75862, indicando que ao menos uma parte do Fe presente nas folhas-bandeira foi mobilizada antes do estágio R5 para outros órgãos, provavelmente os grãos. As duas cultivares com baixa concentração de Fe nos grãos (Canastra e Epagri 108 - em torno de 10 ppm) parecem ter diferentes passos limitantes para o acúmulo de Fe. Canastra apresentou altas concentrações de Fe nas folhas-bandeira no estágio R9, mostrando que o Fe não foi mobilizado em estágios anteriores. A cultivar Epagri 108 apresentou as menores concentrações de Fe nas folhas-bandeira no estágio R9. Entretanto, acreditamos que isso não seja resultado de uma intensa mobilização de Fe, visto que esta cultivar apresenta baixas concentrações de Fe em toda a planta (grãos, raízes e principalmente partes aéreas), mesmo em condição ótima de disponibilidade de Fe (Silveira et al. 2007). Assim, nossos resultados sugerem que baixas concentrações de Fe em grãos de arroz podem ser o resultado de baixos níveis de absorção de Fe e transporte para as folhas-bandeira (como visto na Epagri 108) ou um baixo nível de mobilização de Fe da folha-bandeira (como visto na Canastra). A alta concentração de

Fe em folhas-bandeira (no estágio R9) e a baixa concentração de Fe nos grãos da cultivar Canastra sugerem que a mobilização de Fe das folhas-bandeira é um potencial passo limitante no transporte deste metal até os grãos em desenvolvimento, como já sugerido por Waters e Grusak (2008). Em nossos experimentos verificamos que a concentração de Zn diminui em folhas-bandeira ao longo do desenvolvimento reprodutivo, indicando que o Zn pode ser mobilizado das folhas-bandeira.

Expressão de *OsNAC5* durante a senescência e dependência de ativação por ABA

A remobilização de nutrientes para os grãos já foi associada com o processo de senescência foliar em trigo (Uauy et al. 2006b). Através de experimentos de senescência induzida por estresses, foi visto que a expressão do gene *OsNAC5* aumenta gradualmente após exposição de discos foliares ao escuro. Esta indução é abolida na presença de BAP (6-benzilaminopurina, um inibidor de senescência) e aumentada na presença de ABA (ácido abscísico, um indutor de senescência). Também foi constatado que a expressão de *OsNAC5* é dependente de ABA, pois além de sua indução ser abolida na presença de NA (Nicotinamida, um conhecido inibidor das respostas mediadas por ABA - Leckie et al. 1998), sua região promotora apresenta 10 elementos ABRELATERD1 (ACGTG), típico de regiões promotoras de genes induzidos por ABA (Abe et al. 2003). Analisando 24.209 promotores de arroz (Morris et al. 2008, http://www.bioinformatics2.wsu.edu/Osiris), foi visto que a média é de 2.31 elementos ABRELATERD1 por promotor, mostrando que esse elemento está super-representado no promotor do gene *OsNAC5*. Outros estresses também foram testados e notou-se que a expressão de *OsNAC5* é induzida após exposição das plantas de arroz ao frio e alta salinidade, dois estresses que são relacionados a produção de ABA (Cheng et al. 2009). *OsNAC6,* que pertence a mesma subfamília de *OsNAC5* (*SNAC*), também tem sua expressão induzida por frio, alta salinidade e exposição a ABA (Ohnishi et al. 2005). Entretanto, o aumento na expressão de *OsNAC5* só foi detectado após exposição prolongada a esses estresses (4 dias), sugerindo que a expressão desse gene é induzida somente após o início de um processo de senescência causado por esses estresses, e não diretamente pelo estresse. Após 4 dias, um processo de senescência bem estabelecido

pode ser detectado através do aumento na expressão do gene *OsSGR* (*staygreen*, um marcador de senescência - Park et al. 2007).

OsNAC5 não é induzido por deficiência de Fe em folhas de arroz

De acordo com Ogo et al. (2006), a expressão do gene *OsNAC5* é aumentada em partes aéreas de arroz após curta exposição a deficiência de Fe. Entretanto, em nossos experimentos a expressão desse gene só foi aumentada após longa exposição a esse estresse, quando o processo de senescência já estava bem estabelecido. A análise da expressão do gene *OsIRO2* (Ogo et al. 2006) foi usada para confirmar que as plantas estavam sob deficiência de Fe. Para a análise da expressão gênica de *OsNAC5*, Ogo et al. utilizaram a técnica de microarranjo e nós utilizamos a técnica de PCR em tempo real. De acordo com Schmittgen e Livak (2008), a sensibilidade da técnica de PCR em tempo real é muito maior que a de microarranjos, o que reforça a relevância dos nossos resultados.

Possível relação entre a expressão de *OsNAC5* em folhas-bandeira e a concentração de Fe, Zn e proteínas nos grãos

Foi detectada correlação positiva entre a expressão de *OsNAC5* em folhas-bandeira nas fases R3 (emergência da panícula) e R4 (antese) e a concentração de Fe, Zn e proteínas nos grãos maduros. Na fase R5 (enchimento do grão, quando a maior parte da mobilização de nutrientes deve ocorrer) não foi detectada correlação significativa. Entretanto, a importância da mobilização de nutrientes em estágios iniciais do desenvolvimento reprodutivo foi demonstrada em trigo. A expressão do gene NAM-B1, um fator de transcrição NAC responsável pela regulação da senescência foliar e mobilização de proteínas, Zn, Fe e Mn para os grãos (Uauy et al. 2006b; Distelfeld et al. 2007) é detectada logo após a antese (Uauy et al. 2006b). Além disso, após a aplicação foliar de Zn, a maior concentração desse metal é encontrada no início do desenvolvimento do grão (em torno de 10 dias após a antese ou no início da fase de enchimento do grão), sugerindo que a aplicação foliar de Zn em fases iniciais do desenvolvimento reprodutivo do trigo pode ser uma forma efetiva de aumentar a

concentração de Zn nos grãos através da eficiente mobilização a partir das folhas (Ozturk et al. 2006). Diversos processos essenciais ocorrem em fases iniciais do desenvolvimento, incluindo ramificação da panícula, diferenciação da palea e lema, microsporogênese, polinização e logo depois a antese. Os metais captados por essas estruturas em estágios iniciais do desenvolvimento (R3 e R4) poderiam ser transportados para os grãos em estágios posteriores. De fato, Jiang et al. (2008) mostraram uma menor taxa de absorção de Zn em plantas de arroz durante o enchimento do grão se comparado com estágios anteriores. Estruturas verdes (fotossinteticamente ativas), como a palea e a lema nos estágios R3 e R4, certamente necessitam de Fe em estágios iniciais do desenvolvimento, e esse Fe poderia ser remobilizado durante o processo de senescência dessas estruturas. Além disso, já foi mostrado que um grande acúmulo de Zn em fases iniciais do desenvolvimento dos grãos possivelmente esteja relacionado com a síntese de proteínas. A síntese de proteínas nos grãos é particularmente alta durante as fases iniciais do desenvolvimento (Greene, 1983; Martre et al. 2003) e Zn é o micronutriente que mais afeta a síntese protéica em plantas (Obata et al. 1999). Ainda, de acordo com Lu et al. (2008), existe um QTL em arroz (qZn-11) responsável por 18,61% da variação na concentração de Zn nos grãos. Verificamos que o gene *OsNAC5* localiza-se dentro desse QTL. Nossos resultados mostraram correlação positiva entre as concentrações de Fe, Zn e proteínas nos grãos de arroz. Dessa forma, a mobilização simultânea de Fe, Zn e aminoácidos em fases iniciais do desenvolvimento dos grãos não é inesperada.

A correlação positiva entre a expressão do gene *OsNAC5* em folhas-bandeira durante as fases iniciais do desenvolvimento reprodutivo e as concentrações de Fe, Zn e proteínas nos grãos de arroz sugerem que a expressão deste gene tem uma influência positiva no transporte desses metais e aminoácidos das folhas-bandeira para os grãos.

Outros estudos a serem realizados com o gene *OsNAC5* e a família *SNAC* (*stress-responsive NAC genes*)

A identificação e caracterização inicial do gene *OsNAC5* abriu caminho para novos experimentos que irão confirmar se este gene realmente está envolvido no processo de mobilização de metais para os grãos de arroz. Alguns desses experimentos já vêm sendo realizados pelo nosso grupo e incluem a fusão da região promotora de

OsNAC5 com GFP (para confirmar que sua localização subcelular corresponde ao esperado para proteínas que funcionam como fatores de transcrição), a superexpressão desse gene em plantas de *Arabidopsis* e arroz (para verificar se uma maior expressão desse gene pode levar a uma maior mobilização de metais para os grãos) e a análise de plantas de arroz mutantes (para verificar se a ausência de uma cópia funcional desse gene influencia na concentração de Fe e Zn nos grãos). Uma vez que o gene *OsNAC5* está envolvido com processos de senescência, não sabemos se esse gene causará ou não uma senescência anterior e morte precoce da planta ao invés de proporcionar uma maior mobilização de metais.

Outros genes da família *SNAC* vêm sendo caracterizados pelo nosso grupo quanto à participação em processos de senescência induzido por escuro, na presença de indutores e inibidores de senescência. Também estamos analisando em quais tecidos esses genes são expressos (durante a fase vegetativa e reprodutiva), além de verificar quais hormônios vegetais (etileno, ácido jasmônico e ácido abscísico) são capazes de induzir a expressão desses genes. Linhagens de *Arabidopsis* superexpressando dois desses genes (*ONAC103* e *ONAC068*) serão analisadas quanto a tolerância ao frio e alta salinidade, uma vez que a expressão dos genes correspondentes aumenta após exposição das plantas de arroz a esses estresses (Fang et al. 2008).

Distribuição de Fe nos grãos de diferentes cutivares de arroz

Através de técnicas histoquímicas, não foi possível detectar Fe^{+2} nem Fe^{+3} no endosperma dos genótipos de arroz avaliados. A ausência de Fe no endosperma já havia sido vista anteriormente, e nesses estudos a localização de Fe se restringiu a camada de aleurona e ao embrião (Prom-u-thai et al. 2003; Sivaprakash et al. 2006; Sellappan et al. 2009). Entretanto, Takahashi et al. (2009), utilizando a técnica de fluorescência por raio-x, identificaram Fe também no endosperma. Em nossos experimentos, a presença de Fe^{+2} foi observada somente no embrião de duas cultivares com alto teor de Fe no grão, não sendo detectada na camada de aleurona de nenhuma cultivar testada. A presença de Fe^{+3} foi detectada em todas as cultivares, com maior predominância no embrião. A cultivar IR69428, que possui a maior concentração de Fe no grão, apresentou os maiores níveis de Fe^{+3} no embrião. Na camada de subaleurona, baixas concentrações de Fe^{+3} foram detectadas em todas as cultivares, exceto Canastra, que

possui a menor concentração de Fe no grão. A forma prevalente de Fe encontrada nos grãos das cultivares analisadas (Fe^{+3}) pode estar presente em grânulos de fitina (Inoue et al. 2009) ou estocada na cavidade central da proteína ferritina. Entretanto, já foi demonstrado que as ferritinas de arroz provavelmente estejam mais envolvidas com proteção contra o estresse oxidativo do que com estocagem de Fe no grão (Stein et al. 2009), assim como em *Arabidopsis*, onde somente 5% do Fe total da semente está estocado na ferritina (Ravet et al. 2009). A identificação das proteínas mais importantes para a estocagem de ferro em grãos de arroz poderia ser obtida com a combinação de técnicas de proteômica com técnicas de detecção de metais em amostras de proteína do tipo LA-ICP-MS (Becker et al. 2008).

Análise da expressão de genes relacionados à homeostase de Fe e/ou Zn em folhas-bandeira

Visto que o gene *OsNAC5* é induzido por processos de senescência e que diversos genes que codificam proteínas transportadoras de metais são induzidos durante a senescência foliar de *Arabidopsis* (Van der Graaff et al. 2006), é possível que o fator de transcrição OsNAC5 regule proteínas transportadoras similares em arroz, e que esses genes sejam necessários para a eficiente mobilização de Fe e Zn das folhas-bandeira para os grãos de arroz. Assim, analisamos a expressão de 24 genes sabidamente ou potencialmente envolvidos com a homeostase de Fe e/ou Zn em folhas-bandeira de oito cultivares de arroz (com níveis contrastantes de Fe e/ou Zn nos grãos) em dois estágios do desenvolvimento: R3 (emergência da panícula) e R5 (enchimento do grão) (Counce et al. 2000).

A concentração de Fe nos grãos apresentou correlação positiva com a expressão de *OsYSL14* em folhas-bandeira no estágio reprodutivo R3 e correlação negativa com *OsNRAMP8* e *OsFRO1* em R3 e com *OsNRAMP7* em R5. A concentração de Zn nos grãos apresentou correlação positiva com a expressão de *OsYSL14*, *OsNRAMP1* e *OsNAS1* em R3 e correlação negativa com *OsYSL6*, *OsNRAMP8* e *OsFRO1* em R3 e com *OsYSL8*, *OsNRAMP7* e *OsNAS1* em R5. Essas análises nos permitiram identificar quais genes podem contribuir positiva ou negativamente para o processo de mobilização de Fe e/ou Zn das folhas-bandeira para os grãos de arroz.

A família YSL (*yellow stripe like*) de transportadores é constituída por proteínas capazes de transportar metais conjugados com NA (nicotianamina) através de membranas celulares, e existem evidências de que essas proteínas participam do transporte a longa distância de metais-NA, principalmente Fe (Curie et al. 2009; Ishimaru et al. 2010). A expressão de três genes dessa família (*OsYSL6*, *8* e *14*) apresentou correlação com as concentrações de Fe e/ou Zn nos grãos. Foi sugerido que o produto do gene *OsYSL14* estaria envolvido com o movimento intracelular de metais, uma vez que é expresso apenas em partes aéreas (Nishizawa, 2006). A correlação negativa encontrada para os genes *OsYSL6* e *OsYSL8* poderia ser explicada pelo possível papel das proteínas YSL no movimento de metais para o vacúolo. Dessa forma, uma maior expressão desses genes resultaria em maiores proporções de Fe e Zn indisponíveis para serem transportados para os grãos. AtYSL4 e AtYSL6 foram identificados em um estudo de proteínas de *Arabidopsis* presentes no tonoplasto (Jaquinod et al. 2007), e segundo Curie et al. (2009), AtYSL4 e AtYSL6, juntamente com OsYSL5 e OsYSL6, formam um *cluster* separado na família YSL, sugerindo que as proteínas de arroz também poderiam ser localizadas no tonoplasto e desempenhar a função de transportar os conjugados metais-NA para o vacúolo (Curie et al. 2009).

A expressão do gene *OsNAS1* apresentou correlação positiva com a concentração de Zn nos grãos em R3 e correlação negativa com a concentração de Zn nos grãos em R5. A enzima nicotianamina sintase (NAS) é necessária para a biossíntese de NA, co-substrato das proteínas YSL (Roberts et al. 2004; Schaaf et al. 2004). Durante o transporte no floema, Fe e Zn são potencialmente quelados por NA (von Wirén et al. 1999) e genes NAS já foram mostrados como sendo expressos em células envolvidas com transporte de Fe a longas distâncias (Inoue et al. 2003). Também foi visto que a superexpressão de genes NAS de cevada em tabaco aumenta a concentração de Fe e Zn em folhas jovens, flores e grãos das plantas transgênicas (Takahashi et al. 2003).

A família NRAMP (*natural resistance-associated macrophage protein*) desempenha importante papel na homeostase de metais em diferentes espécies, de bactérias a humanos (Nevo e Nelson, 2006), e sabe-se que estas proteínas transportam uma ampla variedade de substratos (Curie et al. 2000). A expressão de *OsNRAMP1* apresentou correlação positiva com a concentração de Zn nos grãos, e *OsNRAMP7* e *8* apresentaram correlação negativa entre a sua expressão e as concentrações de Fe e Zn nos grãos. OsNRAMP1 pertence a mesma classe de AtNRAMP1, comprovadamente

um transportador de Fe (Curie et al. 2000). A proteína AtNRAMP3 localiza-se no tonoplasto (Thomine et al. 2003) sendo OsNRAMP7 o homólogo de arroz mais próximo de AtNRAMP3. Verificamos que o gene *OsNRAMP7* localiza-se dentro de um QTL previamente identificado por outros grupos, que explica aproximadamente 14% da variação na concentração de Fe e 13% da variação na concentração de Zn nos grãos de arroz (Stangoulis et al. 2007; Garcia-Oliveira et al. 2009). Entretanto, ainda não se sabe se as proteínas NRAMP de arroz participam no influxo ou efluxo de metais no vacúolo. De acordo com nossas análises de correlação, OsNRAMP1 funcionaria no efluxo, exportando metais do vacúolo para o citoplasma, resultando numa maior concentração de metais disponíveis para serem transportados para os grãos. OsNRAMP7 e 8 funcionariam no influxo de metais para o vacúolo, com efeito oposto sobre a alocação desses metais para o grão.

Em arroz, nenhuma atividade de Fe^{+3}-quelato redutase (codificada por genes FRO) é detectada na superfície das raízes (Ishimaru et al. 2006). Em folhas, *OsFRO1* é expresso sob deficiência de Zn, Mn e Cu (Ishimaru et al. 2006). Entretanto, conseguimos detectar altos níveis de expressão de *OsFRO1* em folhas-bandeira de plantas de arroz crescendo em condições normais, o que indica a necessidade de passos de redução para o transporte interno de metais. A expressão do gene *OsFRO1* apresentou correlação negativa com as concentrações de Fe e Zn nos grãos. De acordo com Jeong et al. (2008), AtFRO7 participa na aquisição de Fe pelo cloroplasto. Análises filogenéticas mostraram que OsFRO1 e AtFRO7 são intimamente relacionados (Vinícius de Abreu Waldow, comunicação pessoal). Assim, OsFRO1 poderia desempenhar papel semelhante em arroz, direcionando o Fe para o cloroplasto e impedindo sua mobilização para os grãos. Devido a grande importância do Fe para a fotossíntese, esse direcionamento não seria inesperado. Ainda, uma das principais formas de estocagem de Fe em plantas ocorre dentro dos plastídeos, em proteínas de estocagem de Fe chamadas ferritinas (Zancani et al. 2007). Sabe-se que a redução do Fe^{+3} é um passo necessário para a subsequente estocagem na ferritina (Laulhere e Briat, 1993).

Não há dúvida de que o processo de mobilização de metais das folhas-bandeira para os grãos é extremamente complexo e depende da ação de um número muito maior de genes do que os analisados em nosso estudo. Além disso, a falta de correlação entre a expressão de um gene em folha-bandeira e a concentração de metais nos grãos não exclui esse gene como sendo um dos participantes nesse processo. A análise de outras

cultivares ou de um número maior de cultivares poderia revelar outras correlações significativas. Por último, mas não menos importante, é a extrapolação entre expressão gênica em nível de mRNA e proteína. Devido a mudanças pós-transcricionais, os níveis de expressão de mRNA podem não ser mantidos em nível de proteína ou em nível de atividade enzimática. Dessa forma, consideramos que nosso estudo é um importante avanço no entendimento do mecanismo de mobilização de metais para os grãos, porém, sabemos que representa apenas um ponto de partida para outros estudos funcionais (como análise de plantas mutantes e ensaios de complementação de leveduras mutantes para o transporte de Fe e Zn) que irão elucidar quais desses genes são realmente importantes para a translocação de Fe e/ou Zn para os grãos de arroz.

Biofortificação de arroz com Fe e Zn: qual o melhor caminho?

Existem diversas alternativas para diminuir o problema da deficiência de micronutrientes em humanos: ingestão de uma dieta diversificada (consumo de carne, vegetais, peixes e frutas), suplementação alimentar (ingestão de micronutrientes na forma de tabletes) e fortificação alimentar (adição de nutrientes em alimentos processados). Porém, a efetividade dessas alternativas a longo prazo depende de contínuo apoio financeiro, infraestrutura adequada e uma boa rede de distribuição (White e Broadley, 2009; Goméz-Galera et al. 2010). Uma alternativa viável é a biofortificação, definida como o processo de aumento da biodisponbilidade de nutrientes nas partes comestíveis das plantas (White e Broadley, 2005). A biofortificação pode ser atingida através de fertilização, melhoramento tradicional ou engenharia genética. A fertilização mineral pode ser problemática devido ao alto custo, aderência dos minerais a partículas do solo e questões ambientais (Rengel et al. 1999). Assim, tem aumentado muito o interesse no desenvolvimento de plantas com altas concentrações de micronutrientes essenciais, como Fe e Zn. A principal vantagem da biofortificação por melhoramento genético é que somente é necessário investimento na fase de pesquisa e desenvolvimento (Goméz-Galera et al. 2010).

Diversos grupos de pesquisa já tentaram aumentar as concentrações de Fe e Zn nos grãos de arroz através do aumento na absorção de nutrientes pelas raízes, aumento na taxa de transporte via xilema e floema, aumento na força de dreno do grão e diminuição nos níveis de substâncias que diminuem a disponibilidade dos nutrientes.

Entretanto, muitos aspectos da homeostase de Fe e Zn em arroz permanecem pouco entendidos, e a maior parte das tentativas de biofortificação levou a um pequeno aumento na concentração desses metais nos grãos de arroz, bem abaixo do que certamente é possível. Através de técnicas de melhoramento tradicional, cultivares com maiores concentrações de Fe e Zn e menores concentrações de substâncias inibidoras da disponibilidade destes nutrientes nos grãos poderiam ser cruzadas com cultivares que apresentam alta produtividade. A identificação de QTLs é de extrema importância em programas de cruzamento, e ao passo que mais informaçãoes a respeito dos processos moleculares envolvidos no acúmulo de minerais são desvendados, a modificação genética pode ser aplicada mais facilmente (Tucker, 2003). Entretanto, a diversidade genética das cultivares existentes seria o fator limitante e abordagens envolvendo manipulação genética seriam necessárias para se ter um aumento efetivo e significante nas concentrações de Fe e Zn. Alguns exemplos dessas abordagens são a inserção de novos genes, aumento na expressão de genes já existentes mas pouco expressos, e redução na expressão de genes envolvidos na síntese de inibidores da absorção de Fe e Zn (Lonnerdal, 2003). De qualquer forma, a biofortificação de arroz deve focar o endosperma, a parte mais interna do grão de arroz, que é normalmente consumida e que possui baixíssimos níveis de Fe e Zn (White e Broadley, 2009).

Apesar de a manipulação genética ser uma opção bastante viável para a biofortificação de arroz, ainda existe a necessidade de se identificar a maioria dos mecanismos e proteínas necessárias para o transporte de Fe e Zn para os grãos de arroz. Aumentos expressivos nas concentrações de certos minerais somente serão conseguidos através da inserção de múltiplos transgenes expressos *in tandem* e em estágios de desenvolvimento específicos, para que seja possível uma maior absorção e translocação para os tecidos-alvo (Grusak, 2002; Waters e Grusak, 2008). Provavelmente esses genes não incluam apenas genes responsáveis pela absorção de Fe e Zn, mas também genes que codificam transportadores internos e fatores de transcrição que regulam a expressão de diversos genes envolvidos no processo.

Portanto, ainda há um longo caminho a ser percorrido, tanto na descoberta de novos genes e processos chave na alocação de minerais para o grão de arroz, quanto na construção de plantas transgênicas, em que a eficácia de cada uma das abordagens proposta possa ser testada.

Considerações Finais

Está bem estabelecido na literatura o papel essencial que as folhas-bandeira desempenham em relação à mobilização de fotoassimilados via floema até os grãos. Entretanto, a importância da translocação de Fe e Zn das folhas-bandeira para os grãos ainda é uma suposição. Nossos trabalhos são os primeiros que tentam mostrar a importância das folhas-bandeira nesse processo. Acreditamos que mesmo que existam outros processos mais importantes para o acúmulo de metais nos grãos (como remobilização de outras partes da planta, contínua absorção pelas raízes, transporte direto via xilema), é muito improvável que ao menos uma pequena parte do Fe e Zn encontrado nos grãos não seja proveniente das folhas-bandeira. Dessa forma, mesmo que as folhas-bandeira não sejam a principal fonte de metais para os grãos em desenvolvimento, a remobilização de metais das folhas-bandeira ainda é um processo que merece ser estudado e melhor entendido. A identificação de genes importantes nesse processo pode fornecer alvos para futuros programas de melhoramento tradicional ou transgenia, visando o aumento nas concentrações de Fe e Zn nos grãos de arroz.

Como perspectivas para a continuidade do primeiro trabalho, destacamos a importância da elucidação do papel da proteína OsNAC5 em relação à remobilização de Fe e Zn das folhas-bandeira para os grãos. Para tanto, a superexpressão deste gene em plantas de arroz e de *Arabidopsis* vem sendo realizada, além de experimentos de localização subcelular através de fusão com GFP. A análise de uma linhagem de arroz mutante para este gene também poderá fornecer informações valiosas a respeito de sua função. A análise de correlação entre a expressão deste gene em folhas-bandeira e as concentrações de Fe e Zn nos grãos deve ser realizada utilizando-se um número muito maior de cultivares, para obtermos dados mais robustos. Também seria importante verificar se a proteína OsNAC5 é produzida e induzida mais fortemente em cultivares de arroz com maior concentração de Fe e Zn nos grãos. A produção de anticorpo policlonal e a análise por *western blot*, além de estudos de proteômica comparativa, poderiam responder essas questões.

Como perspectivas para a continuidade do segundo trabalho, destacamos a necessidade de confirmação do envolvimento dos genes que apresentaram correlação significativa entre expressão (em folhas-bandeira) e concentração de Fe e Zn (nos grãos). Para tanto, um número muito maior de cultivares deveriam ser testadas. Mutantes com perda-de-função nos genes encontrados deveriam ser analisados, além de

experimentos de superexpressão em arroz e *Arabidopsis*. A expressão heteróloga em levedura dos seis genes que codificam possíveis transportadores de metais (*OsYSL6*, *OsYSL8*, *OsYSL14*, *OsNRAMP1*, *OsNRAMP7*, *OsNRAMP8*) também deverá fornecer informações sobre a capacidade de transporte de Fe e Zn, e a fusão das regiões promotoras desses genes com GFP indicará a localização subcelular dessas proteínas.

Esperamos que a obtenção desses dados permita lançar alguma luz sobre a função *in planta* desses genes e contribuir para uma melhor compreensão da remobilização de Fe e Zn e do transporte desses metais para os grãos de arroz.

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Apêndices

Outros artigos publicados (ou submetidos) durante o período do doutorado

- Ricachenevsky FK, **Sperotto RA**, Menguer PK, Sperb ER, Lopes KL, Fett JP. ZINC-INDUCED FACILITATOR-LIKE family in plants: lineage-specific expansion in monocotyledons and conserved genomic and expression features among rice (*Oryza sativa*) paralogs. *BMC Plant Biology*, submetido **(apêndice 1).**

- Ricachenevsky FK, **Sperotto RA**, Menguer PK, Fett JP. Identification of Fe-excess-induced genes in rice shoots reveals a WRKY transcription factor responsive to Fe, drought and senescence. *Molecular Biology Reports*, no prelo, 2010 **(apêndice 2).**

- **Sperotto RA**, Ricachenevsky FK, Stein RJ, Waldow VA, Fett JP. Iron stress in plants: dealing with deprivation and overload. *Plant Stress*, no prelo, 2010 **(apêndice 3).**

- Silveira VC, Fadanelli C, **Sperotto RA**, Stein RJ, Basso LA, Santos DS, Vaz Junior IS, Dias JF, Fett JP. Role of ferritin in the rice tolerance to iron overload. *Scientia Agrícola* 66: 549-555, 2009 **(apêndice 4).**

- **Sperotto RA**, Boff T, Duarte GL, Fett JP. Increased senescence-associated gene expression and lipid peroxidation induced by iron deficiency in rice roots. *Plant Cell Reports* 27: 183-195, 2008 **(apêndice 5).**

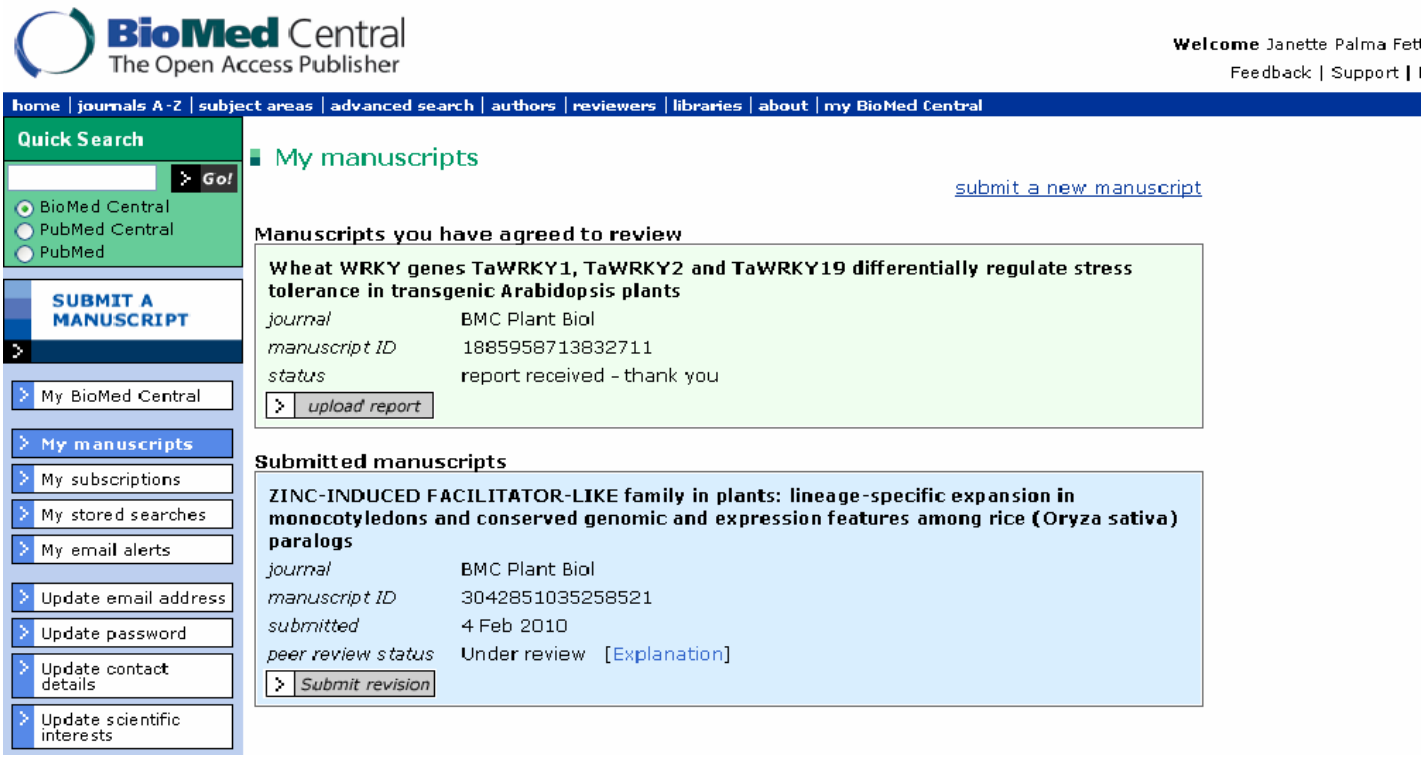
- **Sperotto RA**, Ricachenevsky FK, Fett JP. Iron deficiency in rice shoots: identification of novel induced genes using RDA and possible relation to leaf senescence. *Plant Cell Reports* 26: 1399-1411, 2007 **(apêndice 6).**

- Silveira VC, Oliveira AP, **Sperotto RA**, Espindola LS, Amaral L, Dias JF, Cunha JB, Fett JP. Influence of iron on mineral status of two rice (*Oryza sativa* L.) cultivars. *Brazilian Journal of Plant Physiology* 19: 127-139, 2007 **(apêndice 7).**

Capítulo de livro publicado

- **Sperotto RA**, Ricachenevsky FK, Waldow VA, Fett JP. Iron Biofortification in Rice: It's a Long Way to the Top. *In*: Devarajan Thangadurai (Org.). *Molecular Agrobiology*. Benthan Science Publishers, no prelo, 2010 **(apêndice 8).**

Apêndice 1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Apêndice 2

Mol Biol Rep
DOI 10.1007/s11033-010-0027-0

Identification of Fe-excess-induced genes in rice shoots reveals a WRKY transcription factor responsive to Fe, drought and senescence

Felipe Klein Ricachenevsky · Raul Antonio Sperotto · Paloma Koprovski Menguer · Janette Palma Fett

Received: 22 January 2010 / Accepted: 24 February 2010

Abstract Fe participates in several important reactions in plant metabolism. However, Fe homeostasis in plants is not completely understood, and molecular studies on Fe-excess stress are scarce. Rice (*Oryza sativa* L. ssp. *indica*) is largely cultivated in submerged conditions, where the extremely reductive environment can lead to severe Fe overload. In this work, we used representational difference analysis (RDA) to isolate sequences up-regulated in rice shoots after exposure to Fe-excess. We isolated 24 sequences which have putative functions in distinct cellular processes, such as transcription regulation (*OsWRKY80*), stress response (*OsGAP1*, *DEAD-BOX RNA helicase*), proteolysis (*oryzain-α, rhomboid protein*), photosynthesis (*chlorophyll a/b binding protein*), sugar metabolism (*β glucosidase*) and electron transport (*NADH ubiquinone oxireductase*). We show that the putative WRKY transcription factor *OsWRKY80* is up-regulated in rice leaves, stems and roots after Fe-excess treatment. This up-regulation is also observed after dark-induced senescence and drought stress, indicating that *OsWRKY80* could be a general stress-responsive gene. To our knowledge, this is the first report of an Fe-excess-induced transcription factor in plants.

Keywords Fe-excess · Representational difference analysis · Rice · Stress · WRKY transcription factor

Abbreviations

ABA	Abscisic acid
BAP	6-Benzylaminopurine
CAB	Chlorophyll *a/b* binding protein
DP	Differential product
LRR	Leucine-rich repeat receptor
MES	2,4-Morpholino-ethane sulfonic acid
PSII	Photosystem II
RDA	Representational difference analysis
ROS	Reactive oxygen species
RuBisCO	Ribulose 1,5-bisphosphate carboxylase/ oxygenase
SAG	Senescence-associated gene
SGR	Staygreen
TF	Transcription factor

Introduction

Iron (Fe) is a transition metal essential for virtually all living organisms. Because of its singular capacity to gain or lose electrons, it has a crucial participation in metabolic processes such as oxy-reductive reactions of photosynthesis, respiration and nitrogen assimilation. Fe is very abundant in the soil, but is normally chelated to organic matter in oxidized and insoluble forms, which are not readily available to absorption by plants [1]. Fe-deficiency leads to chlorosis, decreased abundance of photosynthetic proteins and senescence [2–4], being a yield-limiting factor with major implications for field crop production in many

Electronic supplementary material The online version of this article (doi:10.1007/s11033-010-0027-0) contains supplementary material, which is available to authorized users.

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Apêndice 3

MANUSCRIPTS ACCEPTED OR IN PRESS, INCLUDING ABSTRACTS (2010)

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Updated 1st June, 2010 (ordered alphabetically, unless in press)

Plant Stress

SPECIAL ISSUES: PLANT NUTRITION AND ABIOTIC STRESS TOLERANCE (Guest Editor: Naser A. Anjum (Aligarh Muslim University, India)) ~ 2010

Raul Antonio Sperotto, Felipe Klein Ricachenevsky, Ricardo José Stein, Vinicius de Abreu Waldow, Janette Palma Fett (Brazil) Iron Stress in Plants: Dealing with Deprivation and Overload

ABSTRACT

Invited Review: Iron (Fe) is an essential nutrient for plants and one of the most abundant elements in soils. However, it is nearly inaccessible to plants because of its poor solubility in aerobic conditions at neutral or basic pH, resulting in much lower concentrations than required for the optimal growth of plants. However, when Fe is taken up in excess of cellular needs, it becomes highly toxic, since both Fe^{2+} and Fe^{3+} can act as catalysts in the formation of hydroxyl radicals, which are potent oxidizing agents that may damage DNA, proteins and lipids. Plants must be able to sense and respond to Fe stress in terms of both Fe-deprivation and Fe-overload. Depending on the level of severity, plants are unable to deal with such stress and undergo dramatic changes in cellular metabolism with a sequential dismantling of cellular structures, resulting in growth inhibition and ultimately plant death. Therefore, plants must tightly regulate Fe levels within the cell to ensure that Fe is present at adequate levels. Here, we describe recent progress made in understanding how Fe is sensed by plants, and how plants are affected by and try to deal with non-optimal Fe concentrations.

Apêndice 4

Note

ROLE OF FERRITIN IN THE RICE TOLERANCE TO IRON OVERLOAD

Vivian Chagas da Silveira[1]; Cristina Fadanelli[2]; Raul Antonio Sperotto[1]; Ricardo José Stein[1]; Luiz Augusto Basso[5]; Diógenes Santiago Santos[5]; Itabajara da Silva Vaz Junior[1,3]; Johnny Ferraz Dias[4]; Janette Palma Fett[1,2]*

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ABSTRACT: Plants ordinarily face iron (Fe) deficiency, since this mineral is poorly available in soils under aerobic conditions. Nonetheless, wetland and irrigated rice plants can be exposed to excess, highly toxic Fe. Ferritin is a ubiquitous Fe-storage protein, important for iron homeostasis. Increased ferritin accumulation resulting from higher Fe availability was shown in some plant species. However, the role of ferritin in tolerance mechanisms to Fe overload in rice is yet to be established. In this study, recombinant rice ferritin was expressed in *Escherichia coli,* producing an anti-rice ferritin polyclonal antibody which was used to evaluate ferritin accumulation in two rice (*Oryza sativa* L.) cultivars, either susceptible (BR-IRGA 409) or tolerant (EPAGRI 108) to Fe toxicity. Increased ferritin mRNA and protein levels resulting from excess Fe treatment were detected in both cultivars, with higher ferritin protein accumulation in EPAGRI 108 plants, which also reached lower shoot Fe concentrations when submitted to iron overload. The tolerance mechanism to excess Fe in EPAGRI 108 seems to include both restricted Fe translocation and increased ferritin accumulation. This is the first work that shows higher accumulation of the ferritin protein in an iron-excess tolerant *Oryza sativa* cultivar, providing evidence of a possible role of this protein in iron tolerance mechanisms.
Key words: PIXE, anti-ferritin antiserum, metal tolerance

PAPEL DA FERRITINA NA TOLERÂNCIA DE ARROZ AO EXCESSO DE FERRO

RESUMO: Deficiência de ferro (Fe) ocorre freqüentemente em plantas, uma vez que este mineral é pouco disponível em condições aeróbicas. Plantas de arroz cultivadas sob alagamento, no entanto, estão sujeitas ao excesso de Fe, que pode ser extremamente tóxico. Alguns cultivares de arroz são resistentes a altas concentrações de ferro, mas os mecanismos fisiológicos responsáveis por essa resistência são pouco conhecidos. A ferritina é uma proteína de ampla distribuição e capaz de armazenar ferro, sendo considerada importante para a homeostase deste metal. Acúmulo de ferritina em condições de alta disponibilidade de ferro já foi descrito em algumas espécies vegetais. Entretanto, o papel da ferritina no mecanismo de tolerância de plantas de arroz ao excesso de ferro não é conhecido. Neste trabalho, expressamos ferritina de arroz em *E. coli*, produzimos um anticorpo policlonal anti-ferritina de arroz e este foi utilizado para avaliar o acúmulo de ferritina em dois cultivares de arroz (*Oryza sativa*) considerados suscetível (BR-IRGA 409) e tolerante (EPAGRI 108) ao excesso de ferro. O anticorpo foi capaz de reconhecer ferritina purificada de sementes de ervilha, assim como ferritina de folhas de arroz. Aumentos nos níveis de mRNA e proteína foram observados nos dois cultivares sob excesso de ferro, com maior acúmulo da proteína no cultivar EPAGRI 108. Quando submetidas a excesso do elemento, plantas deste mesmo cultivar atingiram concentrações de Fe mais baixas do que plantas do cultivar BR-IRGA409, principalmente nas partes aéreas. Sugere-se que o mecanismo de tolerância ao excesso de ferro no cultivar EPAGRI 108 inclui limitação da translocação de Fe e aumento do acúmulo de ferritina. Este é o primeiro trabalho que mostra maior acúmulo da proteína ferritina em um cultivar de *Oryza sativa* tolerante ao excesso de Fe, fornecendo evidência de um possível papel desta proteína nos mecanismos de tolerância a este metal.
Palavras-chave: PIXE, anti-soro anti-ferritina, tolerância a metal

Apêndice 5

Plant Cell Rep (2008) 27:183–195
DOI 10.1007/s00299-007-0432-6

BIOTIC AND ABIOTIC STRESS

Increased senescence-associated gene expression and lipid peroxidation induced by iron deficiency in rice roots

Raul Antonio Sperotto · Tatiana Boff · Guilherme Leitão Duarte · Janette Palma Fett

Received: 22 May 2007 / Revised: 6 July 2007 / Accepted: 2 August 2007 / Published online: 24 August 2007

Abstract Iron deficiency is among the most common nutritional disorders in plants. Low iron supply causes decreased root growth and even plant death. However, there are no reports about the specific pathways that lead Fe-deficient roots to senescence and death. To investigate the molecular mechanisms that regulate rice roots response to Fe-deficiency, rice seedlings were grown for 3, 6 and 9 days in the presence or absence of Fe. Sequences of 28 induced genes in rice roots under Fe-deficiency were identified by representational difference analysis (RDA). About 40% of these sequences have been previously reported as senescence-related. Differential expression of selected genes was confirmed by semi-quantitative RT-PCR analysis. Classical senescence-related sequences, such as MYB and WRKY transcription factors, cysteine protease, ubiquitin-conjugating enzyme, lipid transfer protein, fatty acid hydroxylase, β-glucosidase and cytochrome P450 oxydoreductase were identified. Fe-deficiency also resulted in decreased dry weight, increased lipid peroxidation (detected by TBA and histochemical methods) as well as evident membrane damage in Fe-deficient roots. Taken together, the results indicate that Fe-deficiency in roots is linked to typical senescence pathways, associated with lipid peroxidation.

Keywords Iron deficiency · Lipid peroxidation · Representational difference analysis · Rice · Root senescence

Abbreviations

bHLH	Basic helix-loop-helix
β-Gluc	Beta-glucosidase
DP	Differential product
ERP	Ethylene-responsive protein
LTP	Lipid transfer protein
MDA	Malondialdehyde
P450	Cytochrome P450 oxydoreductase
RDA	Representational difference analysis
SAG	Senescence-associated gene
SAP	Senescence-associated protein
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid-reagent substances
TCA	Trichloroacetic acid
UBC	Ubiquitin-conjugating enzyme
USP	Universal stress protein

Introduction

Iron (Fe) is necessary for all living cells and plants play a major role in its entry into the food chain. As a transition metal, its ability to gain and lose one electron confers important properties for redox reactions, taking part in proteins essential for photosynthesis, respiration and many other cellular functions, including DNA synthesis and hormone production. Although abundant in soil, Fe is mainly present as oxidized Fe^{3+} compounds, which are poorly soluble in neutral-to-alkaline soils. Consequently,

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Apêndice 6

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BIOTIC AND ABIOTIC STRESS

Iron deficiency in rice shoots: identification of novel induced genes using RDA and possible relation to leaf senescence

Raul Antonio Sperotto · Felipe Klein Ricachenevsky · Janette Palma Fett

Received: 14 November 2006 / Revised: 5 February 2007 / Accepted: 18 February 2007 / Published online: 9 March 2007

Abstract Rice plants are highly susceptible to Fe-deficiency. Under nutrient deprivation, plant cells undergo extensive metabolic changes for their continued survival. To provide further insight into the pathways induced during Fe-deficiency, rice seedlings were grown for 3, 6 and 9 days in the presence or absence of Fe. Using RDA (Representational Difference Analysis), sequences of 32 induced genes in rice shoots under Fe-deficiency were identified. About 30% of the sequences found have been previously reported as responsive to other abiotic and even biotic stresses. However, this is the first report that indicates their relation to Fe deprivation. Differential expression of selected genes was confirmed by semi-quantitative RT-PCR analysis. The identification of classical senescence-related sequences, such as lipase EC 3.1.1.-, ubiquitin-conjugating enzyme EC 6.3.2.19, β-Glucosidase EC 3.2.1.21 and cysteine synthase EC 2.5.1.47, besides the higher accumulation of total soluble sugars prior to the decrease of total chlorophyll content in Fe-deficient leaves, indicate that sugar accumulation may be one of the factors leading to premature leaf senescence induced by Fe-deficiency.

Keywords Iron deficiency · Representational difference analysis · Rice · Leaf senescence

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Abbreviations

ADF	Actin-depolymerizing factor
Alb3	Albino3
β-Gluc	Beta-glucosidase
DMA	Deoxymugineic acid
DP	Differential product
FRO2	Ferric reductase oxidase 2
GDC	Glycine decarboxylase
HEXBP	Hexamer-binding protein
IRT	Iron regulated transporter
LHC	Light harvesting complex
MA	Mugineic acid
NAAT	Nicotianamine amino transferase
NRAMP	Natural resistance-associated macrophage protein
PC	Phytochelatin
PCS	Phytochelatin synthase
PSI	Photosystem I
RAC	Rubisco activase
RDA	Representational difference analysis
SHMT	Serine hydroxymethyltransferase
SucSynth	Sucrose synthase
THF	Tetrahydrofolate
TPI	Triose-phosphate isomerase
TRAPP	Transport protein particle
TSS	Total soluble sugars
UBC	Ubiquitin-conjugating enzyme
YS	Yellow stripe
ZIP	Zinc regulated/iron regulated transporter protein

Introduction

Iron is an essential micronutrient for almost all living organisms, and plants play a major role in its entry into the

Apêndice 7

RESEARCH ARTICLE

Influence of iron on mineral status of two rice (*Oryza sativa* L.) cultivars

Vivian C. da Silveira[1], Anna P. de Oliveira[2], Raul A. Sperotto[1], Luciana S. Espindola[3], Lívio Amaral[3], Johnny F. Dias[3], João B. da Cunha[3] and Janette P. Fett[1,4]*

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Iron is an essential nutrient for plants. In aerobic conditions, Fe is highly unavailable for plant uptake, and Fe deficiency can be severe in plants grown in calcareous soils. In waterlogged soils, however, Fe availability increases and can reach toxic concentrations. Rice is an important staple crop worldwide and faces iron deficiency or excess, depending on the growth conditions. To contribute to the study of mechanisms involved in response to Fe deficiency and resistance to Fe excess, experiments were carried out with rice cultivars BR-IRGA 409 (I409, susceptible to Fe toxicity) and EPAGRI 108 (E108, resistant to Fe toxicity) grown in culture solutions and submitted to Fe excess, control concentration or deficiency (500, 6.5 or zero mg L^{-1} Fe, respectively). Analysis of shoot dry weight confirmed the resistance of E108 plants to Fe excess. Mössbauer spectroscopy analysis indicated the presence of four different Fe^{3+}compounds. The parameters obtained match those expected for ferrihydrite, lepidocrocite (and/or citrate) and Fe-nicotianamine. Mineral concentrations were determined using the PIXE (Particle Induced X-Ray Emission) technique. E108 plants had lower Fe concentrations than I409 plants when exposed to excess Fe. Except for lower Mn levels in roots and shoots, the excess of Fe did not result in lower nutrient concentrations in the susceptible cultivar compared to the resistant one. I409 plants seem to be affected directly by Fe toxicity rather than by secondary effects on mineral nutrition, whereas E108 plants seem to make use of the avoidance mechanism in the resistance to Fe overload. Both cultivars responded to Fe deficiency with allocation of P from roots to shoots. In addition to being more resistant to iron overload, E108 plants seem to be more efficient in inducing Fe deficiency responses.

Key words: ferritin, iron deficiency, iron toxicity; Mössbauer spectroscopy, *Oryza sativa*, PIXE

Influência do ferro no status mineral de duas cultivares de arroz (*Oryza sativa* L.)**:** O ferro é um nutriente essencial para as plantas. Em condições aeróbicas, é altamente indisponível para absorção pelas plantas, e sua deficiência pode ser severa em plantas cultivadas em solos calcáreos. Em solos alagados, no entanto, a disponibilidade de Fe aumenta e pode atingir concentrações tóxicas. O arroz é uma cultura básica mundialmente importante e enfrenta deficiência ou excesso de Fe, dependendo das condições de cultivo. Para contribuir com o estudo dos mecanismos envolvidos nas respostas a deficiência de Fe e resistência ao excesso de Fe, foram realizados experimentos com as cultivares de arroz BR-IRGA 409 (I409 – sensível à toxidez por Fe) e EPAGRI 108 (E108 – resistente à toxidez por Fe) submetidas a excesso de ferro, concentração-controle ou deficiência (500, 6.5 ou zero mg L^{-1} Fe, respectivamente). A avaliação do peso seco das partes aéreas confirmou a resistência da cultivar E108 ao excesso de Fe. A espectroscopia Mössbauer indicou a presença de quatro diferentes compostos de Fe^{3+}. Os parâmetros obtidos coincidem com os esperados para ferrihidrita, lepidocrocito

Abbreviations: DW – dry weight; E108 – EPAGRI 108 rice cultivar; I409 – BR-IRGA 409 rice cultivar; MA – mugineic acid; NA – nicotianamine; PIXE – Particle Induced X-Ray Emission; PS – phytosiderophore

Apêndice 8

Data: Thu, 20 Aug 2009 02:06:40 +0530
De: Devarajan Thangadurai <drdthangaduraibp@gmail.com>
Reponder para: Devarajan Thangadurai <drdthangaduraibp@gmail.com>
Assunto: Molecular Agrobiology - Instructions for Authors - reg.
Para: cooolaiya@yahoo.com

Dear Contributing Authors,

Greetings, due to overwhelming response from the scientific community, we are unable to accommodate all the accepted articles in the proposed book, "*Plant
Biotechnology and Transgenic Research*". Hence it has decided by the editors and the publisher to publish all other remaining articles in an another forthcoming book entitled "*Molecular Agrobiology*". This book will also be published by the Bentham Science Publishers, USA. As one of the editors, I am happy to share with you here that these 43 articles have been critically evaluated, considered and accepted from the bunch of more than 150 articles considered for publication.

Please find the list of review articles finally approved and accepted for publication in our forthcoming book entitled "*Molecular Agrobiology*". Also attached herewith is the instruction for manuscript preparation and submission. Moreover, the last date for the submission of article in complete has been extended up to 30.11.2009.

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Janette Palma Fett

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Iron biofortification in rice plants: A long way to the top

***CURRICULUM VITAE* resumido**

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4. PRÊMIOS E DISTINÇÕES

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