Universidade Federal do Rio Grande do Sul – UFRGS
Programa de Pós-Graduação em Biologia Celular e Molecular - PPGBCM
Centro de Biotecnologia
Laboratório de Fisiologia Vegetal

Identificação de genes ativados por deficiência de ferro em partes aéreas de arroz (*Oryza sativa* L. ssp. *indica*)

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ABREVIATURAS

- *At = Arabidopsis thaliana*
- ATP = trifosfato de adenosina
- bHLH = basic helix-loop-helix
- cDNA = DNA complementar
- DMA = ácido desoximuginéico
- EROs = espécies reativas de oxigênio
- Fer = ferritina
- FIT1 = Fe-deficiency induced transcription factor 1
- FRO = ferric reductase oxidase
- FUR = FER-like regulator of iron uptake
- GUS = gene repórter (β-Glicuronidase)
- H_2O_2 = peróxido de hidrogênio
- IDS2 = iron deficiency-specific clone 2
- IRO2 = iron-related transcription factor 2
- IRT1 = iron regulated transporter
- ITP = iron transport protein
- *Le = Lycopersicon esculentum*
- LHC = light harvesting complex (complexo de captação de luz)
- MA = ácido muginéico
- mRNA = RNA mensageiro
- NA = nicotianamina
- NAAT = nicotianamina amino transferase
- NAS = nicotianamina sintase
- NRAMP = natural resistance-associated macrophage protein
- OPT = oligopeptide transporter (transportador de oligopeptídeo)
- *Os = Oryza sativa*
- PCR = polymerase chain reaction (reação em cadeia da polimerase)
- *Ps = Pisum sativum*
- PS = fitossideróforo
- RDA = representational difference analysis (análise de diferenças representacionais)

- SAM = S-adenosil metionina
- WHO = World Health Organization (Organização Mundial da Saúde)
- YS = yellow stripe
- ZIP = Zinc Regulated Transport / Iron Regulated Transport-like Protein
- *Zm* = *Zea mays*

INTRODUÇÃO GERAL

Características gerais do ferro e sua homeostase em arroz

O ferro é um elemento essencial para o desenvolvimento das plantas, uma vez que é parte integrante de diversas enzimas que participam de processos metabólicos muito importantes como respiração, fotossíntese, fixação do nitrogênio, síntese de clorofila, entre outros. O ferro é conhecido como um metal de transição, pois é capaz de receber e doar elétrons, ou seja, caracteriza-se pela facilidade em mudar seu estado de oxidação. Por este motivo, está envolvido em diversas reações de transferência de elétrons. Devido a esta capacidade, o ferro pode formar complexos com diversos ligantes. Em sistemas aeróbicos, o ferro 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 ferro 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 arroz cultivado, *Oryza sativa*, pertence à família Poaceae. É considerada 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). É também a planta modelo para estudos de fisiologia e genética em monocotiledôneas (Shimamoto e Kyozuka, 2001), além de ser a terceira maior cultura de grãos no mundo, perdendo apenas para milho e trigo. No Brasil, a produção é de aproximadamente 13 milhões de toneladas anuais, o que faz do país o décimo maior produtor mundial, sendo considerado 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 responsável por 46% da produção nacional (aproximadamente seis milhões de toneladas) na safra 2004/2005.

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 ferro no vegetal. O ferro em excesso é extremamente tóxico, uma vez que pode agir como catalisador na formação de EROs. Através da reação de Fenton, o Fe^{+2} reage com H_2O_2 levando à oxidação do ferro (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). 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 ferro são o bronzeamento das folhas, 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 ferro pode levar a perdas de 15 a 30 % na lavoura de arroz, dependendo da região e da cultivar plantada (Lopes, 1987).

Embora o ferro seja 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 ferro forma polímeros de óxido-hidróxidos altamente insolúveis. A concentração de ferro 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 ferro é um grande problema para plantas que crescem em solos calcáreos, que representam 1/3 de todos os solos cultiváveis. A deficiência de ferro 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 (Larcher, 2003). 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 captação de ferro e a distribuição dentro dos vários órgãos da planta (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 and Terry, 1980), além de diminuição na expressão da Rubisco, de proteínas que se ligam à clorofila e enzimas envolvidas na síntese deste pigmento (Spiller et al. 1987; Winder and Nishio, 1995; Belkhodja et al. 1998). Esta clorose está associada à inibição na síntese de clorofila, cuja biossíntese é realizada por enzimas que contêm ferro. Entretanto, Moseley et al. (2002) descreveram em *Chlamydomonas reinhardtii* uma série de respostas do aparato fotossintético frente à deficiência de ferro que ocorrem antes da clorose. Inicialmente ocorre uma desconexão do complexo de captação de luz I (Light Harvesting Complex I - LHCI) do fotossistema I (PSI), seguida de um remodelamento deste complexo (degradação específica de proteínas já existentes e síntese de novas proteínas), diminuindo a capacidade de transferência de elétrons destes complexos. Assim, o aparato fotossintético é otimizado e os danos foto-oxidativos são minimizados através de respostas graduais. A atividade do PSI é muito mais afetada por deficiência de ferro que o PSII. Suprindo uma planta clorótica com quantidades normais de ferro, a função do PSI como transmissor de elétrons aumenta muito mais que a do PSII. Nessa condição, os componentes individuais do PSI, P700, citocromos e proteínas aumentam, indicando que o ferro está envolvido na regulação da montagem do PSI e na junção das subunidades na membrana do tilacóide (Pushnik e Miller, 1989). Se a deficiência de ferro se torna mais severa, a atividade do PSII também cai drasticamente e é muito mais difícil de ser restaurada (Morales et al. 1991). 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 and 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). A deficiência de ferro tem muito menos efeito no crescimento das folhas, número de células por área, ou número de cloroplastos por célula se comparado com o tamanho dos cloroplastos e o conteúdo de proteínas do cloroplasto. A divisão celular somente é inibida em casos extremos, com deficiência severa de ferro, o que leva à redução no crescimento das folhas

(Abbott, 1967). O ferro é necessário para a síntese de proteínas, e o número de ribossomos diminui em células foliares deficientes em ferro (Lin e Stocking, 1978). Entretanto, sob deficiência de ferro, a síntese de proteínas nos cloroplastos é muito mais prejudicada que no citoplasma (Shetty e Miller, 1966). Em folhas de milho deficientes em ferro, por exemplo, o conteúdo de proteínas totais diminui 25%, mas o conteúdo de proteínas do cloroplasto diminui 82% (Perur et al. 1961). Nas raízes, características morfológicas e fisiológicas são modificadas em situação de deficiência de ferro. Em dicotiledôneas e monocotiledôneas (exceto gramíneas), a deficiência de ferro está associada com inibição do alongamento da raiz, aumento no diâmetro da zona apical da raiz e abundante formação de pêlos radiculares (Römheld e Marschner, 1981; Chaney et al. 1992). Essas mudanças morfológicas são freqüentemente associadas com a formação de células de transferência, tanto na rizoderme como na hipoderme (Landsberg, 1989). A formação de células de transferência induzida por deficiência de ferro é parte de um mecanismo regulatório para aumentar a captação deste metal (Kramer et al. 1980).

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

Estratégias de absorção de ferro

Em condições de suficiência de ferro, 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 ferro, as plantas desenvolveram diferentes estratégias para aumentar a captação deste nutriente, as chamadas estratégias I e II (Marschner and Römheld, 1994).

A estratégia I é utilizada por dicotiledôneas e monocotiledôneas não-gramíneas e envolve a liberação de prótons para a rizosfera, pela ação de uma H^{+}-ATPase, codificada pelo gene *Aha2* em *Arabidopsis* (Fox e Guerinot, 1998), com o intuito de diminuir o pH do solo e aumentar a solubilidade do ferro, seguido da indução de uma Fe^{+3} redutase, codificada pelo gene *FRO2* em *Arabidopsis* (Robinson et al. 1999), cuja função é reduzir Fe^{+3} para a forma mais solúvel Fe^{+2}, e a captação via *IRT1*, um transportador específico de Fe^{+2} (Eide et al. 1996), considerado o transportador de ferro mais importante das raízes (Connolly et al. 2002). Algumas espécies que utilizam a

estratégia I apresentam alterações morfológicas, incluindo o desenvolvimento de pêlos radiculares e células de transferência, enquanto outras secretam flavinas e compostos fenólicos (Marschner et al. 1986; Schmidt e Bartels, 1996; Schmidt et al. 2000).

Provavelmente a redução de Fe^{+3} para Fe^{+2}, ao invés da absorção de Fe^{+2}, seja o passo limitante na captação de ferro em plantas da estratégia I (Connolly et al. 2003). Duas estratégias complementares permitiram a identificação do gene que codifica a Fe^{+3} quelato redutase. Yi e Guerinot (1996) identificaram um mutante de *Arabidopsis* (*frd1*, de ferric reductase defective) incapaz de induzir a atividade de redutase em resposta à deficiência de ferro. Uma estratégia baseada em PCR permitiu a identificação de uma família gênica em *Arabidopsis* (*FRO*, de ferric reductase oxidase) que mostrou homologia com Fe^{+3} quelato redutase de levedura (Robinson et al. 1997). O gene *FRO2* está localizado na mesma posição da mutação *frd1*. Transformação do mutante *frd1* com o gene *FRO2* recuperou o fenótipo selvagem, mostrando que *FRO2* codifica a Fe^{+3} quelato redutase de raiz (Robinson et al. 1999). *FRO2* contém oito domínios transmembrana e quatro resíduos de histidina que provavelmente coordenam dois grupos heme localizados na membrana (Robinson et al. 1999). Acredita-se que *FRO2* funcione recebendo elétrons citosólicos, passando esses elétrons através dos grupos heme presentes na membrana para o Fe^{+3} presente no solo, que é então convertido a Fe^{+2}. Em ervilha, a expressão de *FRO1* é induzida em folhas em resposta à deficiência de ferro e hibridização *in situ* mostrou que *PsFRO1* é expresso nas células do mesófilo das folhas (Waters et al. 2002). A expressão de *AtFRO3* também é induzida em folhas de plantas sob deficiência de ferro (Wintz et al. 2003). *FRO2* de *Arabidopsis* é expresso predominantemente nas células epidérmicas de raízes sob deficiência de ferro, e foi visto que a superexpressão de *AtFRO2* confere à planta a capacidade de crescer em baixas concentrações de ferro (Connolly et al. 2003).

Uma estratégia de complementação funcional de leveduras mutantes permitiu a identificação de *IRT1* (Iron Regulated Transporter), que codifica o principal transportador de alta afinidade responsável pela captação de ferro do solo (Eide et al. 1996; Vert et al. 2002). *IRT1* também é capaz de transportar zinco, manganês, cádmio e cobalto (Eide et al. 1996). Interessantemente, mutações em determinados resíduos de aminoácidos alteram a especificidade do transportador *IRT1* (Rogers et al. 2000). *IRT1* é expresso em células epidérmicas de raízes e flores (Vert et al. 2002) e a expressão em raízes é induzida por deficiência de ferro (Connolly et al. 2002). A perda de atividade de *IRT1* leva à redução no acúmulo de ferro, alteração na morfologia dos cloroplastos,

clorose severa e incapacidade de produzir sementes, a menos que o meio de crescimento seja suplementado com altas concentrações de ferro (Vert et al. 2002). Provavelmente, a aplicação de altas doses de ferro recupera o mutante *irt1* pela ação de transportadores de baixa afinidade presentes em células da camada mais externa da raiz. *IRT1* pertence à família ZIP (Zinc Regulated Transport / Iron Regulated Transport-like Protein) de transportadores de metal, caracterizados por apresentar ampla afinidade para o transporte de vários minerais, principalmente zinco e ferro (Guerinot, 2000). Assim como *IRT1*, *IRT2* também é expresso nas camadas mais externas de raízes sob deficiência de ferro (Vert et al. 2001). Entretanto, a função de *IRT2* ainda não é bem conhecida, uma vez que o mutante *irt2* não apresenta fenótipo diferente do selvagem e superexpressão de *IRT2* no mutante *irt1* não leva à recuperação do fenótipo selvagem (Vert et al. 2002; Varotto et al. 2002). Homólogos de *IRT1* também foram identificados em arroz, uma espécie que utiliza a estratégia II de absorção de ferro (Bughio et al. 2002). Mais tarde, Ishimaru et al. (2006) mostraram que plantas de arroz são capazes de utilizar as duas estratégias de absorção de ferro. Os membros da família ZIP caracterizam-se por ter estrutura conservada, incluindo oito domínios transmembrana e um domínio intracelular entre os domínios transmembrana 3 e 4. Em muitas proteínas ZIP, o domínio intracelular apresenta um motivo de histidinas. Em *IRT1*, o motivo consiste de uma série de resíduos alternados de histidina e glicina (HGHGHGH). O motivo de histidina parece estar envolvido na percepção da quantidade de metal dentro da célula, mas para o motivo de glicina ainda não foi designada nenhuma função (Eide et al. 1996).

Em tomate (*Lycopersicon esculentum*), foi mostrado que o gene *FER* codifica um fator de transcrição básico do tipo hélice-alça-hélice (bHLH) essencial para a ativação dos genes envolvidos na redução do Fe^{+3} e no transporte do Fe^{+2} (Ling et al. 2002), uma vez que mutantes *fer* são incapazes de induzir a expressão de *LeFRO1* e *LeIRT1* nas raízes (Li et al. 2004; Bauer et al. 2004; Brumbarova e Bauer, 2005). Em *Arabidopsis*, foi identificado um ortólogo do gene *LeFER*, chamado *AtFUR* (FER-*like regulator of iron uptake*), que é expresso principalmente nas raízes em condição de deficiência de ferro (Jacoby et al. 2004). O mesmo gene foi identificado em *Arabidopsis* por outro grupo de pesquisa, sendo chamado de *AtFIT1* (*Fe-deficiency induced transcription factor 1*). Foi mostrado que *FIT1* regula a expressão de *FRO2* em nível transcricional (acumulação de mRNA) e *IRT1* em nível pós-transcricional (acumulação de proteína). Por análise de microarranjo, foram identificados 72 genes regulados por

ferro cuja expressão é dependente de *FIT1* (Colangelo e Guerinot, 2004). Em arroz foi recentemente isolado e caracterizado o gene *OsIRO2*, um fator de transcrição básico do tipo hélice-alça-hélice (bHLH), que é fortemente expresso em raízes e partes aéreas sob deficiência de ferro e que está envolvido na regulação da expressão de diversos genes participantes do processo de captação de ferro (Ogo et al. 2006).

As gramíneas utilizam a estratégia II de absorção em situação de deficiência de ferro. Assim como a maioria dos microrganismos, as gramíneas utilizam sideróforos como estratégia para mobilizar e absorver ferro. Os fitossideróforos (PS) são aminoácidos não-proteinogênicos pertencentes à família dos ácidos muginéicos (MA) (Mori, 1999). Estes são liberados pelas raízes no solo, onde são complexados com Fe^{+3} e absorvidos pela planta na forma Fe^{+3}-fitossideróforo via um transportador específico do tipo *YS1* (Yellow Stripe-like transporter) (Curie et al. 2001). Da mesma forma que *IRT1*, *YS1* faz parte de uma família maior de proteínas, compreendendo oito membros presentes no genoma de *Arabidopsis*. Estas plantas podem aumentar a biossíntese e a secreção de PS em resposta à deficiência de ferro. Diferentes espécies de gramíneas produzem diferentes tipos e quantidades de PS. A quantidade de PS liberada no solo correlaciona com a habilidade da planta em tolerar a deficiência de ferro. Plantas de arroz, especialmente nos estágios iniciais de desenvolvimento, são altamente suscetíveis a baixos suprimentos de ferro. Arroz produz muito menos ácido desoximuginéico (DMA) que cevada ou aveia, que podem produzir também outras formas de PS, e por um intervalo de tempo maior (Mori et al. 1991). Provavelmente seja por estas razões que cevada e aveia sejam relativamente resistentes à deficiência de ferro. Takahashi et al. (2001) demonstraram um aumento na captação de ferro em plantas de arroz transformadas com dois genes *NAAT* (nicotianamina amino transferase) de cevada, uma enzima chave no processo de biossíntese de DMA. A presença de inúmeros homólogos YS em dicotiledôneas (que utilizam a estratégia I) tem sugerido que essa família desempenha outros papéis dentro da planta, não somente o de absorção de ferro (Curie et al. 2001).

Os fitossideróforos de plantas foram primeiramente descritos em aveia e arroz (Takagi, 1976). Desde então, a via bioquímica para síntese de PS vem sendo elucidada e diversos genes essenciais foram clonados em cevada e arroz (Takagi et al. 1984; Higuchi et al. 1996; Higuchi et al. 1999; Takahashi et al. 1999; Nakanishi et al. 2000; Yamaguchi et al. 2000; Kobayashi et al. 2001). A biossíntese de PS inicia quando três moléculas de S-adenosilmetionina (SAM) são combinadas para formar uma molécula de

nicotianamina (NA). A enzima nicotianamina sintase (NAS) é a responsável por catalisar esta reação. Higuchi et al. (1999) foram os primeiros a isolar sete genes *NAS* de cevada. Pouco depois, outros genes *NAS* foram descritos em tomate (Ling et al. 1999), *Arabidopsis* (Suzuki et al. 1999), arroz (Higuchi et al. 2001) e milho (Mizuno et al. 2003). A expressão e a atividade de *NAS* nas raízes é regulada através da disponibilidade do ferro. *NAS1* de cevada não é expresso em raízes sob suficiência de ferro, mas é expresso em altos níveis em raízes sob deficiência (Higuchi et al. 1999). Em milho, os transcritos de *NAS1* e *NAS2* são detectados em baixas concentrações em raízes, mas não em folhas de plantas sob suficiência de ferro; entretanto, a expressão é induzida em raízes sob deficiência (Mizuno et al. 2003). Os transcritos de *NAS1* e *NAS2* de arroz, bem como as proteínas codificadas por esses genes, acumulam-se em resposta a baixo suprimento de ferro (Inoue et al. 2003). Análises com o gene repórter *GUS* fusionado aos promotores dos genes *OsNAS1* e *OsNAS2* mostraram que, em condição de suficiência de ferro, esses genes são expressos nas células companheiras das raízes e nas células do periciclo adjacentes ao xilema, enquanto que em deficiência de ferro a expressão desses genes estendia-se a toda raiz, e coincidia com um aumento na secreção de PS.

Até a síntese de NA, os passos para a produção de PS são os mesmos em monocotiledôneas e dicotiledôneas. Os passos subseqüentes são exclusivos de gramíneas. A desaminação da NA é realizada pela enzima nicotianamina amino transferase (NAAT) (Kanazawa et al. 1994). O produto desta reação, ácido 2'-desoximuginéico (DMA), é o precursor para todos os outros ácidos muginéicos (MA). Takahashi et al. (2001) mostraram que a inserção de genes *NAAT* de cevada no genoma de arroz aumenta a secreção de PS e, conseqüentemente, aumenta a tolerância de arroz à baixa disponibilidade de ferro, sugerindo que a conversão de NA para DMA é o passo limitante na produção de MA em arroz. DMA pode ser hidroxilado por uma dioxigenase codificada pelo gene *IDS3* para formar MA. MA e DMA podem sofrer uma hidroxilação adicional catalisada pela proteína IDS2 para formar derivados do tipo epiHMA (ácido 3-epihidroximuginéico) e epiHDMA (ácido 2-epihidroxidesoximuginéico). O grupamento hidroxil adicional parece estar envolvido no aumento da estabilidade do complexo Fe^{+3}-quelante (von Wirén et al. 2000). Os mecanismos moleculares que controlam a secreção de MA para o solo ainda são incertos. Até hoje, nem os genes, nem as proteínas envolvidas no efluxo de MA foram identificados. Nishizawa e Mori (1987) sugeriram que a secreção de MA pode ocorrer

através de transporte vesicular, uma vez que raízes de cevada sob deficiência de ferro apresentam um aumento no tamanho e no número de vesículas nas células corticais, que coincide com o início da liberação de MA.

O processo de captação de Fe^{+3}-PS começou a ser desvendado através da análise do mutante *yellow stripe1* (*ys1*) de milho. Este mutante produz quantidades normais de PS, mas é incapaz de transportar o complexo Fe^{+3}-PS eficientemente (von Wirén et al. 1994). Curie et al. (2001) clonaram o gene *YS1* de milho e mostraram que codifica um transportador de Fe^{+3}-PS localizado nas raízes. *ZmYS1* possui 682 aminoácidos e contém 12 regiões transmembrana. Experimentos de complementação de leveduras mutantes mostraram que a expressão de *ZmYS1* recupera a capacidade do mutante *fet3fet4* (defectivo no transporte de ferro de baixa e de alta afinidade) de crescer em baixas concentrações de ferro quando este é adicionado ao meio na forma Fe^{+3}-DMA, e não na forma Fe^{+3}-citrato (Curie et al. 2001). Análises de expressão gênica mostraram que os transcritos de *ZmYS1* são detectados em raízes, mas não em folhas de plântulas sob condição de suficiência de ferro. Por outro lado, a expressão é induzida em raízes e também em folhas quando as plantas são crescidas na ausência de ferro. Os níveis de proteínas de ZmYS1 coincidem com os níveis de transcritos, uma vez que somente tecidos com baixas concentrações de ferro apresentam altos níveis da proteína ZmYS1 (Roberts et al. 2004). A expressão de *ZmYS1* em folhas sugere que este gene pode ter várias funções na planta, ou seja, além de participar do processo de captação de ferro do solo, pode participar também na distribuição de ferro para as partes aéreas da planta (Curie et al. 2001). Ensaios de complementação funcional de leveduras mutantes, expressão em oócitos de *Xenopus* e captação de isótopos radioativos mostraram que ZmYS1 é capaz de transportar Fe^{+3}-PS, Fe^{+2}-NA e provavelmente Fe^{+3}-NA, o que corrobora a idéia de que ZmYS1 é capaz de transportar os complexos Fe-NA em tecidos de partes aéreas e Fe-PS em raízes de plantas crescidas sob deficiência de ferro (Roberts et al. 2004; Schaaf et al. 2004). Após a identificação de *ZmYS1*, um grande número de ortólogos em arroz, *Arabidopsis* e milho vêm sendo descritos. A presença de genes *YSL* (*yellow stripe-like*) em *Arabidopsis* é bastante intrigante, uma vez que é uma planta que utiliza a estratégia I de absorção de ferro e é incapaz de sintetizar ou utilizar MAs. Em plantas que utilizam a estratégia I, as proteínas YSL funcionam no transporte de metais ligados a nicotianamina no interior da planta (Ling et al. 1999; Curie et al. 2001). Le Jean et al. (2005) mostraram que o gene *YSL1* de *Arabidopsis* (*AtYSL1*) regula os níveis de ferro e nicotianamina nas sementes. Mais tarde, Waters et al. (2006) mostraram que o

duplo mutante de *Arabidopsis ysl1ysl3* apresentava sintomas de deficiência de ferro, como clorose intervenal, além de ter a concentração de ferro nas sementes mais baixa que a planta selvagem. O gene *YSL2* de *Arabidopsis*, ao contrário do gene *YS1* de milho, transporta apenas Fe^{+2} e Cu^{+2} complexado com NA ou MA. Além disso, é expresso em diversos tipos celulares de raízes e partes aéreas, sugerindo que muitos tipos de células obtêm metais na forma complexada com NA ou MA. Baseado no seu padrão de expressão, a função principal de *AtYSL2* parece ser o de transporte lateral de metais no tecido vascular (DiDonato Jr. et al. 2004). O gene *YSL2* de arroz (*OsYSL2*) é expresso em células do floema e é capaz de transportar tanto ferro como manganês na forma complexada com NA, e possivelmente seja responsável pela translocação destes dois nutrientes para os grãos (Koike et al. 2004).

Transporte e compartimentalização do ferro

Depois de absorvido, o ferro é transportado via xilema através da via transpiratória, processo onde participam moléculas quelantes de metal, como o ácido cítrico (Cataldo et al. 1988). Nas folhas, o complexo Fe^{+3}-citrato é reduzido, sendo posteriormente transportado por proteínas carreadoras de cátions bivalentes, através da membrana plasmática. A nicotianamina é um ácido orgânico conhecido por formar complexos com Fe^{+2} tanto no floema como no interior das células. Além do transporte realizado através da complexação com nicotianamina, o ferro 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} (Krüger et al. 2002).

A subseqüente compartimentalização do ferro em organelas, como vacúolos, plastídios e mitocôndrias pode ser realizada por transportadores de metais bivalentes, e sua alocação em diferentes organelas está relacionada com o uso imediato ou armazenamento deste nutriente. A realocação do ferro estocado no interior de vacúolos para as necessidades celulares foi descrita em levedura e viu-se que é parcialmente mediada pelo gene *Smf3p* (Curie e Briat, 2003). Em *Arabidopsis*, foram identificadas cinco proteínas NRAMP. *AtNRAMP3*, um transportador de Fe^{+2} localizado no tonoplasto (membrana do vacúolo), possivelmente esteja relacionado com a remobilização do ferro que encontra-se no vacúolo para o metabolismo celular

(Thomine et al. 2003). Lanquar et al. (2005) mostraram que *AtNRAMP3* e *AtNRAMP4* funcionam mobilizando o estoque de ferro do vacúolo, o que é essencial para a germinação das sementes sob baixa concentração de ferro. Os transcritos de *AtNRAMP1* acumulam-se em resposta à deficiência de ferro em raízes, mas não em folhas (Curie et al. 2000). Belouchi et al. (1997) clonaram e caracterizaram três genes *NRAMP* em *arroz (OsNRAMP1, OsNRAMP2* e *OsNRAMP3*). Experimentos de expressão mostraram que enquanto *OsNRAMP1* é expresso principalmente nas raízes e *OsNRAMP2* em folhas, *OsNRAMP3* é expresso em níveis semelhantes em raízes e folhas. Também foi mostrado que *OsNRAMP1* é capaz de complementar funcionalmente o mutante *fet3fet4* (Curie et al. 2000).

A maior parte do ferro encontrado nas células vegetais localiza-se no interior do cloroplasto, envolvido no processo fotossintético (biossíntese das moléculas de clorofila e ferredoxina). No interior dos plastídios encontra-se a ferritina, proteína globular multimérica de 24 sub-unidades, em cujo interior podem ser estocados até 4500 átomos de ferro (Briat e Lobreaux, 1997). Esta proteína atua como um reservatório celular de ferro (Briat et al. 1999), sendo responsável pelo seu seqüestro e liberação. Quatro genes de ferritina foram identificados no genoma de *Arabidopsis* e cada um codifica uma proteína que contém um peptídeo de trânsito para ser transportada ao plastídio. A expressão de *AtFer1* e *AtFer3* é ativada nas raízes e partes aéreas em resposta ao excesso de ferro e H_2O_2, sugerindo que a complexação do ferro com a proteína ferritina serve para proteger as células do stress oxidativo causado pelo excesso de ferro. A expressão de *AtFer4* é induzida por excesso de ferro, mas não por H_2O_2. A expressão do gene *AtFer2* é específica para síliquas maduras e sementes secas, sugerindo um papel na estocagem de ferro em sementes (Petit et al. 2001).

Estudos de expressão gênica

Um estudo em larga escala realizado em 2001 examinou, através de microarranjo, as mudanças nos níveis de expressão de 16.128 clones de cDNA (correspondendo a pelo menos 6.000 genes) de *Arabidopsis* em resposta à deficiência de ferro. O maior número de clones de cDNA diferencialmente expressos foi observado após 3 dias de tratamento, onde 2.240 clones tiveram sua expressão induzida nas partes aéreas e 776 nas raízes. Também foi vista correlação entre a repressão de enzimas fotossintéticas e o aumento no tempo de exposição à deficiência de ferro (Thimm et al.

2001). Em 2002 foram examinadas, também através de microarranjo, as mudanças nos perfis de expressão de 8.987 clones de cDNA de arroz (aproximadamente um terço de todos os genes) em resposta à deficiência de ferro, sendo utilizado mRNA de cevada na hibridização. Este estudo mostrou que 200 clones foram induzidos nas raízes após duas semanas de exposição ao tratamento, sendo que apenas sete já haviam sido identificados em estudos anteriores (Negishi et al. 2002). Em 2003, novamente por microarranjos, foram identificados novos transportadores de *Arabidopsis* envolvidos na resposta à deficiência de cobre, zinco e ferro, entre eles *AtOPT3*, membro de uma família gênica de transportadores de oligopeptídeos, similar a *YS1* de milho. Foi visto que a expressão deste gene em raízes submetidas à deficiência de ferro era equivalente a do gene *FRO2* (Fe^{+3} redutase) (Wintz et al. 2003). Em 2005 foi analisada, através de microarranjo, a expressão de 8.987 clones de cDNA de arroz em resposta à deficiência de ferro. Foi visto que 57 genes tinham sua expressão modificada, entre eles diversos genes envolvidos em mecanismos de captação de ferro. Também foi verificada a indução dos genes envolvidos em todos os passos do ciclo da metionina, além dos genes necessários à biossíntese de ácidos muginéicos (MAs) a partir de metionina. Assim, verificou-se que a expressão de genes relacionados à aquisição de ferro em condições limitantes é regulada coordenadamente (Kobayashi et al. 2005).

Justificativa e objetivos gerais

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 ferro no genoma do arroz. Quarenta e três genes potencialmente envolvidos na homeostase do ferro (incluindo genes envolvidos nas estratégias I e II de absorção de ferro) foram identificados pelo nosso grupo, com base na semelhança com outros genes relacionados à homeostase do ferro já conhecidos em outras plantas. Foram identificados 18 genes *YSL*, 2 *FROs*, 13 *ZIPs*, 8 *NRAMPs* e 2 *Ferritinas* (Gross et al. 2003). A presença de genes envolvidos na estratégia I de absorção (de não-gramíneas) no genoma do arroz indica que as estratégias não são totalmente exclusivas. O padrão da expressão da maioria destes 43 genes está sendo avaliado com o uso da técnica de macroarranjos de DNA, em plantas de arroz de cultivares sensíveis e resistentes ao excesso de ferro submetidas à deficiência, níveis controle e excesso de ferro. Com esta abordagem espera-se elucidar

quais destes genes são importantes tanto nas respostas à deficiência quanto ao excesso de ferro. Estes poderão ser utilizados tanto como marcadores funcionais no melhoramento tradicional da cultura (Andersen e Lubberstedt, 2003) quanto como possíveis candidatos para transformação genética posterior. No entanto, esta abordagem possui a limitação de avaliar somente a expressão daqueles genes sobre os quais já se suspeita que estejam envolvidos na homeostase do ferro. Outros genes (transportadores, fatores de transcrição, proteínas estruturais, de resposta a stress, entre outros) certamente também são importantes para estes processos, e uma abordagem complementar se faz necessária para que possam ser identificados.

Assim, utilizamos a técnica de RDA (*Representational Difference Analysis*) com o intuito de identificar novos genes envolvidos na homeostase do ferro em arroz, em especial na resposta das plantas à deficiência deste nutriente. Esta técnica foi inicialmente desenvolvida para identificar diferenças entre populações de DNA genômico (Lisitsyn et al. 1993), sendo posteriormente modificada para permitir a análise de diferenças entre populações de mRNAs expressos (Hubank e Schatz, 1994). Baseada em ciclos sucessivos de hibridização subtrativa seguidos de PCR, esta técnica enriquece e permite o isolamento de mRNAs expressos diferencialmente, enquanto, simultaneamente, reduz a representação de seqüências não-diferencialmente expressas. É um método bastante sensível e permite o isolamento de genes cuja expressão é aumentada ou diminuída ao se comparar duas diferentes populações de cDNA (Pastorian et al. 2000), bastando para isso variar as populações "*tester*" e "*driver*". Considerando sua fácil manipulação e eficiência, é uma técnica recomendada para a análise diferencial de expressão gênica em laboratórios de pequeno porte (Kozian e Kirschbaum, 1999).

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Identification of novel genes induced by iron-deficiency in rice (*Oryza sativa* L.) shoots

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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 required for survival during Fe-deficiency, rice seedlings were grown for three, six and nine days in the presence or absence of Fe. Using RDA (Representational Difference Analysis) strategy, thirty two induced genes in rice shoots under Fe-deficiency were isolated. About 30% of the sequences found are responsive not only to Fe-deficiency, but 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 classicall senescence-related sequences, such as lipase EC 3.1.1.-, ubiquitin-conjugating enzyme EC 6.3.2.19 and cysteine synthase EC 2.5.1.47, besides the higher accumulation of total soluble sugars in Fe-deficient leaves, indicates that Fe-deficiency may trigger sugar-induced senescence in rice shoots.

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 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. These same redox properties allow iron to catalyze the formation of damaging oxygen radicals, especially the formation of hydroxyl radicals, the most potent oxidizing agents known (Halliwell and Gutteridge, 1992). These radicals can cause multiple damage to cellular structures such as lipids, proteins and nucleic acids, eventually leading to cell death (Briat et al. 1995). This problem is common for plants growing in anaerobic conditions and acid pH, as found in waterlogged soils, since in these environments the ferrous form is highly soluble and is readily taken up by plants, leading to iron toxicity. On the other hand, under aerobic

conditions and neutral or alkaline pH iron often forms highly insoluble ferric-hydroxide precipitates that limit its availability for plants (Guerinot and Yi, 1994). Therefore, plants need to tightly control iron uptake, intracellular compartmentalization and distribution into the various plant organs (reviewed in Kerkeb and Connolly, 2006).

Among the abiotic stresses, Fe-deficiency constitutes a major factor leading to reductions in crop yield. Fe-deficiency also compromises human health since plants are the most common source of dietary iron. According to the World Health Organization (http://www.who.int/nutrition/publications/micronutrients/en/index.html), iron deficiency is the leading human nutritional disorder worldwide, affecting over 2 billion people. In mammals, Fe-deficiency promotes severe anemia. In photosynthetic organisms, the major symptom is chlorosis (chlorophyll deficiency), mainly on young leaves, which is concomitant with decreased abundance of the photosynthetic machinery (Spiller and Terry, 1980). In this regard, Fe-deficiency stress is correlated with changes in chloroplast ultrastructure (Spiller and Terry, 1980), decreased expression of the small and large subunits of Rubisco, and of chlorophyll *a/b*-binding proteins, as well as decreased chlorophyll synthesis (Spiller et al. 1987; Winder and Nishio, 1995; Belkhodja et al. 1998). Chlorosis was always attributed to inhibition of chlorophyll synthesis, which requires the function of Fe-containing enzymes. However, Moseley et al. (2002) described a series of gradual responses from the photosynthetic apparatus to Fe-deficiency that occurred prior to the onset of chlorosis in *Clamydomonas reinhardtii.* Some responses are the disconnection of the light harvesting complex I (LHCI) antenna from photosystem I (PSI), followed by remodeling of the antenna complex (specific degradation of existing proteins and synthesis of new ones), establishing a new steady state with decreased stoichiometry of electron transfer complexes. In this way, the photosynthetic apparatus is optimized and

the photooxidative damage is minimized in gradual responses. Yet, low iron supply alters protein and lipid composition of thylakoid membranes (Nishio et al. 1985), reduces electron transport capacity in thylakoids (Spiller and Terry, 1980) and diminishes noncyclic ATP formation (Terry, 1980) and leaf ATP levels (Arulanantham et al. 1990), but does not affect the activity of key photosynthetic enzymes as glyceraldehyde-3-phosphate dehydrogenase, fru-1,6-biphosphatase, malic enzyme and PEP carboxylase (Stocking, 1975; Taylor et al. 1982).

To overcome the generally low supply of iron, plants have evolved two distinct strategies, Strategy I and Strategy II, to take up iron from the rhizosphere (Marschner and Römheld, 1994). Strategy I is used by all plants except grasses and involves the release of protons (through the action of a H^+-ATPase) into the rhizosphere to lower the soil pH, the induction of ferric chelate reductase activity of *FRO2* to reduce Fe^{+3} to the more soluble Fe^{+2} form (Robinson et al. 1999), and the uptake of the resulting Fe^{+2} via *IRT1*, an Fe^{+2} specific transporter (Eide et al. 1996), considered the major iron transporter in roots (Connolly et al. 2002). Grasses, a group that includes most of the world's staple grains (*e.g.*, rice, wheat and corn), use the Strategy II response, which relies on chelation of Fe^{+3} rather than reduction. Mugineic acid (MA) family phytosiderophores are released from their roots into the soil where they chelate Fe^{+3} and are absorbed by the plant in the Fe^{+3}-phytosiderophore state via specific *YS1*-like transporters (Curie et al. 2001). These plants can increase the production and secretion of MAs in response to Fe-deficiency. Rice plants, especially at the early stage of growth, are highly susceptible to Fe-deficiency. They produce much lower amounts of deoxymugineic acid (DMA) than barley, which can also produce other forms of phytosiderophores, and for a longer period of time (Mori et al. 1991). Takahashi et al. (2001) have demonstrated improved Fe uptake in rice plants transformed with two

NAAT (nicotianamine amino transferase, EC 2.6.1.80) genes from barley, a critical enzyme in the biosynthesis of DMA. Recently, it was shown that rice plants are able to uptake Fe^{+2} from the rhizosphere through Strategy I, using *OsIRT1* and *OsIRT2* genes (Ishimaru et al. 2006), a strategy that is advantageous for growth in submerged conditions, where Fe^{+2} is more abundant than Fe^{+3}.

During the search for proteins involved with iron mobilization, transport and storage, our group has identified in the rice genome 43 genes potentially related to these processes, comprising five distinct gene families: eighteen *YS1*-like, two *FRO*, thirteen *ZIP* (Zinc Regulated Transporter / Iron Regulated Transporter Protein), eight *NRAMP* (Natural Resistance-Associated Macrophage Protein) and two *Ferritin* genes (Gross et al. 2003). However, other genes that may participate in the response of plants to low Fe supply still remain to be defined. Aiming at better understanding the processes involved in the response of rice shoots to Fe-deficiency stress, we report the identification of new genes responsive to low Fe supply using the RDA (*Representational Difference Analysis*) technique, including senescence-related genes, indicating a link between Fe-deficiency and premature leaf senescence.

Materials and Methods

Plant growth

Rice seeds (*Oryza sativa* L. spp. indica) from BR-IRGA 409 cultivar were provided by IRGA (Instituto Rio-Grandense do Arroz). Seeds were germinated for 4 days in an incubator (28°C, first two days in the dark and last two days in the light) on petry dishes lined with filter paper soaked with distilled water. After germination and growth in vermiculite and nutrient solution (Yoshida et al. 1976) for 14 days (28°C, with 16 hours of light), plants were transferred to glass pots involved in aluminum foil

and containing 500 ml of nutrient solution. Plants (four plants per pot) were kept for 10 days in adaptation solution (modified from Yoshida et al. 1976) containing 3.2 ppm Fe as $FeCl_3.6H_2O$, pH 5.0 ± 0.1. Plants were then transferred to the control condition (6.5 ppm Fe as $FeSO_4.7H_2O$, pH 5.0 ± 0.1) or to the Fe-deficiency treatment (no iron added, with addition of 0.15 mM Ferrozine®, Sigma) for additional 3, 6 and 9 days. All solutions were replaced every 2 days. Plants were cultivated in a growth room at 26 ± 1°C under white light (Osram Universal 20W, K128 B 6, Osasco, Brazil) with a photoperiod of 16 / 8 h light / dark cycle (irradiance of approximately 60 μmol m^{-2} s^{-1}).

RNA extraction and cDNA synthesis

Rice shoot tissues were harvested from plants grown under iron-sufficient or iron-deficient conditions for 3, 6 and 9 days (n = 4 per each group). Total RNA was extracted using Trizol (Gibco BRL, Grand Island, NY, USA). Evaluation of RNA concentration was carried out by measuring absorbance at 260 and 280 nm (Cintra 5 - GBC Scientific Equipment, Victoria, AU) and denaturing agarose gel electrophoresis was used to assess the samples quality. cDNA was prepared using the SMART PCR cDNA Synthesis Kit by Clontech Laboratories (Palo Alto, CA, USA), according to the manufacturer's instructions, in the presence of RNase OUT (Invitrogen Life Technologies). First-strand cDNA synthesis was performed with reverse transcriptase (Superscript II, Invitrogen, CA, USA) using 1 μg of RNA (approximately 300 ng from each time: 3, 6 and 9 days). First-strand cDNA (2 μl) was used as template to synthesize the second strand of cDNA. All RNA preparations were pre-screened for residual DNA contamination by PCR using actin specific primers (Yokoyama et al. 2004).

Representational Difference Analysis

The cDNA Representational Difference Analysis (cDNA-RDA) was performed essentially as described by Hubank and Schatz (1994) and optimized by Pastorian et al. (2000). Double-stranded cDNA pools (approximately 2 μg) were digested with the restriction enzyme Sau3AI (Amershan Pharmacia Biotech, Amershan Place, UK). The resulting products were purified using GFX kit (Amershan Pharmacia Biotech) and ligated to adaptors (RBam12: GATCCTCGGTGA and RBam24: AGCACTCTCCAGCCTCTCTCACCGAG). To provide sufficient amounts of starting material for the RDA, the cDNAs were diluted and amplified using RBam24 as a primer (25 cycles of denaturing at 95°C for 45 sec and annealing and extension at 70°C for 3 min). All the remaining rounds of hybridization used aliquots derived from the same driver cDNA bulk synthesis reaction. The final PCR products were purified using the GFX kit, resulting in driver cDNA populations from shoots submitted to Fe-sufficient and Fe-deficient conditions. To generate the tester cDNA population from Fe-deficient condition, a small aliquot of the driver was completely digested with Sau3AI to remove the 24-mer primers that had been incorporated into the cDNA by PCR. After purification using the GFX kit, the resulting product was ligated to different 24-mer primers (NBam12: GATCCTCCCTCG and NBam24: AGGCAACTGTGCTATCCGAGGGAG). In order to isolate the genes up-regulated in response to Fe-deficiency, we used cDNA from Fe-sufficient (control condition) as driver and Fe-deficient rice shoots as tester.

To perform the first round of hybridization and amplification, generating the first differential product (DP1), driver and tester populations were mixed in the ratio of 50:1 (5 μg driver: 100 ng tester) in a 5 μl reaction at 67°C for 24 hours, and amplified by PCR with NBam24 primer (7 cycles at 95°C for 45 sec and 70°C for 3 min). PCR products were diluted and submitted to a new round of amplification to remove

unwanted single-stranded cDNAs. After purification, DP1 products were digested with Sau3AI to remove NBam adaptors before ligation to JBam adaptors (JBam12: GATCCGTTCATG and JBam24: ACCGACGTCGACTATCCATGAACG). The second hybridization was performed at a ratio of 500:1 (5 μg driver: 10 ng tester). The second differential product (DP2) was obtained using the same procedure as for DP1, using JBam24 as primer, instead of NBam24.

Cloning and sequence analysis

One hundred ng of the final RDA product (DP2 fragments) was purified and cloned into pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen). *Escherichia coli* XL1 Blue competent cells were transformed with the ligated products. Bacterial clones were picked up and grown in 96-well plates. Plasmid DNA was prepared from 300 selected clones and samples were sequenced in the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The vector sequence was removed manually and the resulting sequences were then matched to existing rice full-length cDNA clones using 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).

Semi-quantitative RT-PCR analysis

For the synthesis of first strand cDNA, 1 μg of total RNA from each time of exposure to the treatment (Fe-deficiency or control condition) was reverse transcribed using an oligo-dT_{30} primer and Superscript II (Invitrogen) in 20 μl total volume. The synthesized first strand cDNA was diluted five times and 2 μl were used for PCR

analysis with different sets of gene-specific primers. 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). The PCR conditions used were 95°C for 30 s, 60°C for 30 s and 72°C for 30 s in a 50 µl volume. The numbers of PCR cycles were determined for each gene to ensure that amplification occurred in the linear range. The obtained DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide. The signal intensity of the stained bands was photographed using a digital imager (Kodak DC120 Zoom Digital Camera) and analyzed using Kodak Digital Science 1D Image Analysis Software, Version 3.0. Amplified actin gene was used to normalize the data. For each gene, the sample with the highest normalized optical density was designated 1.00 and the normalized optical densities for the remaining five samples were expressed as percentages.

Total soluble sugars (TSS) content

Samples of about 0.1 g of leaves from rice plants submitted to control or Fe-deficient conditions were homogenised in 2 ml of 80% (v/v) ethanol, maintained at 75°C for 10 min and centrifuged at 10 000 g for 10 min. The insoluble fraction of the extract was washed with 2 ml of 80% ethanol. Both soluble fractions were collected and stored at 4°C for TSS determination. TSS content was quantified according to the method described by Irigoyen et al. (1992). Fifty µl of the alcoholic extract were reacted with 950 µl freshly prepared anthrone (150 mg anthrone + 100 ml 72% [w/w] H_2SO_4) and placed in a boiling water bath for 10 min. After cooling, the absorbance at 650 nm was determined in a Cintra 5 - GBC Scientific Equipment spectrophotometer. The experiment was performed in triplicate and D-Glucose was used as standard.

Statistical analysis

All data were subjected to ANOVA and means were compared by the Duncan test ($P \leq 0.05$). All calculations were made using the SPSS Base 10.0 for Windows (SPSS Inc., USA).

Table 1

Results and Discussion

Isolation of iron-responsive cDNA clones by RDA

The RDA technique was used to identify activated genes in rice shoots submitted to Fe-deficiency. Fig. 1 shows a smear of cDNA products in the starting material. After two cycles of RDA using different tester and driver ratios, a different pattern was evident, especially in the Differential Product 2 (DP2). These resulting RDA fragment populations were cloned into the pCR2.1-TOPO vector and approximately 300 clones with an average length of 200-600 pb were sequenced and analyzed using the Rice Pipeline. After these analyses, 32 putative up-regulated sequences were identified in rice shoots under Fe-deficiency (Table 2).

Fig. 1

Up-regulated genes

The sequences were classified into functional categories, as shown in Table 2. We have detected two sequences involved with photosynthesis: Chloroplast membrane protein ALBINO3 (*Alb3*) and Ribulose-1,5-biphosphate carboxylase activase (*RAC*). The photosynthetic system in higher plants is highly susceptible to abiotic stresses, leading to inhibition of photosynthetic CO_2 assimilation. The energy imbalance between light harvesting and CO_2 assimilation leads to an overexcitation of the photosynthetic apparatus, increasing the potential for photoinhibition and photooxidative damage. To avoid the energy imbalance resulting from abiotic stress, plants are able to down-

regulate genes associated with photosynthetic light harvesting to reduce the light energy absorbed (Huner et al. 1998). This reduction leads to down-regulation of Rubisco (EC 4.1.1.39) activity (Terry, 1980). Moreover, proteomic studies under abiotic stresses in rice already reported degradation of Rubisco subunits (Agrawal et al. 2002; Yan et al. 2006).

Table 2

According to Moseley et al. (2002), adaptation of *Chlamydomonas reinhardtii* to Fe-deficiency requires remodeling of the photosynthetic apparatus, starting with uncoupling of the antenna from the PSI core followed by specific degradation of LHCs, induction of new LHCs, and concluding with assembly of new antenna complexes. The induction of a new polypeptide after exposure to Fe-deficiency was shown by 2-D gel electrophoresis (Moseley et al. 2002). In our experiments, there was up-regulation of *Alb3* gene under Fe-deficiency. The Alb3 protein is required for the integration of the light-harvesting complex (LHC) protein into the thylakoid membrane and it was shown that loss of Alb3 in *Chlamydomonas reinhardtii* leads to reduction (> 10-fold) in the amount of LHCI and LHCII and about 70% reduction in total chlorophyll (Bellafiore et al. 2002; Göhre et al. 2006). It would be very tempting to say that rice, as *Chlamydomonas*, has an “adaptation program” which alters the composition and structure of the LHCs in order to regulate the excitation energy delivery to reaction centers, preventing photodamage, and that Alb3 would be responsible for the assembly of the new and/or adapted photosystem units. However, further studies are necessary to confirm this hypothesis.

Rubisco activase (*RAC*) is a low-molecular-weight ATP-binding protein that modifies the conformation, facilitates the dissociation of inhibitors (tightly bound sugar phosphates) from active sites and increase the enzymatic activity of Rubisco. The induction of *RAC* transcripts and protein level have been shown to be responsive to

several stress conditions such as high temperature in wheat leaves (Law and Crafts-Brandner, 2001), low temperature (Yan et al. 2006), drought (Salekdeh et al. 2002; Ali and Komatsu, 2006) and high salinity (Parker et al. 2006) in rice leaves, besides the feeding of brown planthopper *Nilaparvata lugens* (Homoptera: Delphacidae) on rice plants (Yuan et al. 2005). However, Agrawal et al. (2002) and Wong et al. (2006), respectively, reported a decrease in its expression under ozone stress in rice and under drought and high salinity stresses in *Thellungiela*, a close relative of *Arabidopsis*. The activation of *RAC* observed in our results may be a mechanism to rapidly activate Rubisco in order to enhance its catalytic activity, possibly to compensate for the protein degradation.

In our analysis, we detected five sequences involved in carbohydrate metabolism (Glycine decarboxylase (EC 1.4.4.2), Triose-phophate isomerase (EC 5.3.1.1), Chloroplast β-Glucosidase (EC 3.2.1.21), Sucrose synthase (EC 2.4.1.13) and NADH-ubiquinone oxidoreductase (EC 1.6.5.3) complex I, 21 kDa subunit), suggesting a key role for energy production in response to Fe-deficiency in rice shoots. Glycine decarboxylase (*GDC*) is responsible for the inter-conversion of glycine and serine. L-serine facilitates chlorophyll loss and proteolysis in leaves and its concentration increases in senescing tissues. It was suggested that serine might be incorporated into the active center of one or more proteolytic enzymes participating in the senescence process, thereby allowing increased degradation (Peterson and Huffaker, 1975). However, the synthesis of serine from glycine and vice versa is not the only function of GDC. Together with the enzyme serine hydroxymethyltransferase (SHMT, EC 2.1.2.1), GDC converts tetrahydrofolate (THF) into N^5,N^{10}-methylenated, making these reactions the most important source of active one-carbon-units for a number of biosynthetic processes, such as the biosynthesis of methionine, pyrimidines, purines, chlorophyll and

glutathione (Bauwe and Kolukisaoglu, 2003). Besides, *GDC* participates in photorespiration and therefore is important for the maintenance of the electron flow, preventing photoinhibition under stress conditions (Wingler et al. 2000). Increased *GDC* expression in rice leaves under low temperature has been reported (Yan et al. 2006). Triose-phosphate isomerase (*TPI*) plays important roles in triose-phosphate-involving metabolic cascades in plants, such as gluconeogenesis, fatty acid biosynthesis, pentose phosphate pathway and photosynthetic carbon dioxide fixation (Miernyk, 1990). Riccardi et al. (1998) showed the induction of *TPI* in response to progressive water deficit in maize leaves; Thimm et al. (2001) showed that *TPI* and other genes involved in glycolysis are induced in *Arabidopsis* shoots submitted to Fe-deficiency stress and Salekdeh et al. (2002) showed its induction in rice leaves under drought. β-Glucosidase (*β-Gluc*) is responsible for hydrolysis of terminal, non-reducing β-D-glucose residues with release of β-D-glucose and its activation has been reported under several abiotic stresses such as water deficit in maize (Riccardi et al. 1998); cold, salt and drought in *Arabidopsis* (Kawasaki et al. 2001; Kreps et al. 2002; Seki et al. 2002); cold acclimation in *Arabidopsis* (Fowler and Thomashow, 2002) and salinity in *Thellungiella* (Wong et al. 2006), besides biotic stresses such as the blast fungus infection in rice cell suspension-cultures (Kim et al. 2003) and brown planthopper infestation in rice leaves (Wang et al. 2005). Sucrose synthase (*SucSynth*) converts sucrose and UDP into fructose and UDP-glucose. Fructose is converted into hexose phosphates and then channeled into glycolysis and the pentose phosphate pathway. The UDP-glucose produced by *SucSynth* is a glucose donor for the synthesis of polysaccharides such as cellulose, hemicelluloses and starch. In *Arabidopsis*, this gene is up-regulated by low temperature (Fowler and Thomashow, 2002; Seki et al. 2002) and in *Thellungiella* its expression is drought- and cold-induced (Wong et al. 2006). Bleecker and Patterson

(1997) proposed that the decline of photosynthetic activity below a certain threshold level could result in a decline in photosynthetic metabolites, such as sugars, thereby activating senescence pathways. Yuan et al. (2005) concluded that sugar starvation may be one of the factors activating leaf senescence in rice infected with the homoptera *Nilaparvata lugens*. Yoshida (2003) suggested that senescence may be induced by sugar depletion in some species. However, there has been some debate whether leaf senescence is induced by sugar starvation or by sugar accumulation. Sugar content tipically increases during *Arabidopsis* leaf senescence (Diaz et al. 2005) and Pourtau et al. (2006) showed an increase in accumulation of the hexoses glucose and fructose in senescent leaves, concluding that *Arabidopsis* leaf senescence is induced rather than repressed by sugars. Here, we investigated how endogenous sugar content change during control and Fe-deficiency treatments through the analysis of total soluble sugars content (Fig. 2). Sugars reached maximum values in leaves exposed for nine days to Fe-deficiency treatment, suggesting that Fe deprivation leads to higher sugar accumulation, which may play an important role in the establishment of senescence.

Fig. 2

One of the differentially expressed sequences found matches with a *COBRA*-like protein/phytochelatin synthase (*PCS*, EC 2.3.2.15). *COBRA*-like are glycosylphosphatidylinositol-anchored proteins that play an important role in the biosynthesis of cell walls of mechanical support tissues. Membrane deterioration is an early and characteristic feature of senescence that results in increased permeability, loss of ionic gradients and decreased activities of key membrane-associated enzymes. Mutation in *BC1*, which encodes a *COBRA*-like protein, causes not only a reduction in cell wall thickness and cellulose content, but also an increase in lignin levels (Li et al. 2003). Leuchter et al. (1998) reported the complementation of a *Saccharomyces pombe* mutant defective in phytochelatin synthesis using a partial *COBRA*-like cDNA from

Arabidopsis. Although phytochelatin synthesis activity may not be directly related to *COBRA*-like function, it is possible that this protein binds divalent metal, as part of a "buffering" system to protect against damage caused by heavy-metals. *PCS* catalyzes the synthesis of phytochelatin (PC) from reduced glutathione. PCs belong to a family of peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where *n* equals 2-11 and can form stable heavy metal complexes that are subsequently sequestered from the cytosol into vacuoles, avoiding toxic effects on metabolism. In this way, PCs play a central role in heavy metal tolerance and detoxification of Cd^{+2} and other toxic metals (Clemens, 2006). An increase in expression of phytochelatin synthase (PCS) was shown in *Arabidopsis* leaves under Fe-deficiency. However, the increase detected was very subtle, which is consistent with the known role of PCs in detoxifying excess metals rather than involvement in metal deficiencies (Wintz et al. 2003). On the other hand, Cohen et al. (1998) observed that Fe-deficiency elicits a large stimulation of Cd^{+2} and Zn^{+2} influx and accumulation in roots but not in shoots of pea seedlings and Cu^{+2} and Mn^{+2} in both roots and shoots, suggesting the necessity of a scavenger mechanism to chelate and detoxify these metal ions. Another explanation for the increased expression seen in phytochelatin synthase under Fe-deficiency could be the chelation of metal ions released during cellular degradation promoted by proteolytic enzymes. In this way, *PCS* might participate in protecting the cellular integrity required for progression and completion of senescence. Leaf senescence is a developmentally programmed degeneration process that constitutes the final step of leaf development and is controlled by multiple developmental and environmental signals. Rather than being a disorganized breakdown of all cellular components, it is highly regulated and involves a series of ordered events, including the recycling of valuable nutrients to other parts of the plant

(Lim et al. 2003). Therefore, maintenance of cellular integrity and of energy supply are necessary for its successful completion.

We detected up-regulation of the gene encoding an ubiquitin-conjugating enzyme in our experiments, as previously reported in the literature (Genschik et al. 1994), that indicates the activation of polyubiquitin and ubiquitin-conjugating enzymes during senescence. Garbarino et al. (1995) showed that expression of a marker gene transcribed from a polyubiquitin promoter was 5- to 10-fold higher in senescent leaves relative to young leaves. The ubiquitin pathway also plays a role in recycling nitrogen through proteolysis of unnecessary proteins and possible utilization of aminoacids as another source of energy, to maintain vital functions in the senescent cells (Courtney et al. 1994). Ubiquitin-conjugating enzymes (*UBCs*) catalyze the second step in the ubiquitin-dependent proteolytic pathway, one of the major protein degradation pathways in all eukaryotes. *UBCs* are induced under stress conditions such as heat shock and heavy-metal treatment. The stress-induced *UBCs* are suggested to be responsible for selective degradation of proteins folded incorrectly as a result of stress (Garbarino et al. 1995).

Lipid degradation is one of the biochemical events of cellular senescence. It has been reported that β-oxidation increases in plants during carbon starvation (Dieuaide et al. 1992). The 3-ketoacyl-CoA thiolase (EC 2.3.1.16) gene from pumpkin (*Cucurbita* sp.) was characterized by Kato et al. (1996). It is responsible for the last step in the oxidation of β-fatty acids and its expression increases during cotyledon senescence, coinciding with the transition of microbodies from peroxisomes to glyoxysomes. Also, Contento et al. (2004) showed that transcript levels for a peroxisomal acyl-CoA synthase (EC 2.3.1.86) and a peroxisomal acyl-CoA oxidase (EC 1.3.3.6, involved in short-chain acyl-CoA oxidation) increased in cell suspension cultures of *Arabidopsis*

under sucrose starvation. In our experiments, we isolated a GDSL-motif lipase, suggesting an increase in the breakdown of fatty acids, possibly to provide energy for the senescing leaf.

Four structural proteins were detected in our experiments, Bet3-transport protein particle (TRAPP) component, Dynamin-like protein (EC 3.6.5.5), Centromere protein-like and Actin-depolymerizing factor 5. *Bet3*, the most highly conserved component of the TRAPP (transport protein particle) complex, plays a key role in ER-to-Golgi transport (Sacher et al. 1998). *Dynamin* is a high molecular weight GTP-binding protein that is known to constrict membranes and to facilitate the release of vesicles from the plasma membrane and the Golgi apparatus, also being involved in various steps of intracellular trafficking, such as endocytosis, vacuole trafficking, mitochondria morphology and fission, and chloroplast trafficking. It is considered a membrane-deforming protein (Hinshaw, 2000). The actin cytoskeleton is a dynamic network in all eukaryotic cells. It is involved in a variety of morphogenetic events and responds to biotic and abiotic stimuli. The actin filaments have the ability to break down into monomeric or G-actin and to reform into filamentous or F-actin. This dynamic process is regulated by actin-depolymerizing factor (*ADF*) genes. The induction of *ADF* transcripts was shown in rice plants submitted to drought, high-salinity and abscisic acid exposure (Rabbani et al. 2003), barley seedlings under salinity stress (Walia et al. 2006) and barley roots submitted to Fe-deficiency (Negishi et al. 2002). Increased ADF protein accumulation was shown in rice leaves during drought stress (Salekdeh et al. 2002; Ali and Komatsu, 2006).

Two sequences were placed on the protein-protein interaction functional category, the WWE domain-containing CEO protein and the Chloroplast heat shock protein-binding protein. CEO is a plant-specific protein which is able to protect yeast

against oxidative damage in complementation experiments (Belles-Boix et al. 2000). This protein contains a conserved globular domain called the WWE domain, which is predicted to mediate protein-protein interactions with transcription factors, as revealed by yeast two-hybrid analysis (Belles-Boix et al. 2000). Such analysis suggests that CEO might be involved in regulating gene expression by affecting the function or activity of transcription factors (Ahlfors et al. 2004). Based on the nature of transcription factors that interact with CEO, Belles-Boix et al. (2000) suggested that this protein may participate in the mechanism of adaptation to biotic and abiotic stress conditions as a cofactor of transcription factors.

Aquaporins are membrane proteins whit the ability to facilitate and regulate passive exchange of water across membranes. However, water is not the only molecule capable of transiting through aquaporins. The transport of other small molecules, such as glycerol, urea, ammonia and CO_2 has been reported recently (reviewed in Hachez et al. 2006). It has been reported that *RWC3* (or *OsPIP1;3*) plays a role in drought resistance (Lian et al. 2004) and is induced by brown planthopper in rice (Wang et al. 2005). Apparently, Fe-deficiency is one more stress which results in induction of aquaporins.

We have detected two sequences involved with nucleotide-binding, Hexamer-binding protein family (HEXBP) and SF2/ASF-like pre-mRNA splicing factor. *HEXBP* (hexamer-binding protein) encodes a sequence-specific DNA-binding protein that contains nine cysteine-rich motifs which are identical to a consensus sequence know as the “CCHC type” zinc finger in *Leishmania*. This motif is present in a number of nucleic acid-binding proteins. Proteins containing this motif function by binding to single-stranded nucleic acids (Webb and McMaster, 1993). It will be interesting to investigate the relations between this transcription factor and Fe-deficiency in plants.

We have detected four sequences that can not be placed in one specific functional category: Cysteine synthase, 2-on-2 Hemoglobin, S-adenosylmethionine synthetase (EC 2.5.1.6) and Deoxycytidine/Cytidine deaminase protein (EC 3.5.4.-). Cysteine synthase plays a major role in response to Fe-deficiency and senescence. It catalyzes the formation of cysteine, the only sulfide donor for all reduced sulfur containing cell constituents in plants. Cysteine plays an essential role in both structural and catalytic properties of proteins and, together with iron, is indispensable for electron transfer reactions in photosynthesis and respiration (Hell et al. 2002). It is also involved in the synthesis of methionine (required for continuous synthesis of MAs). Moreover, cysteine accelerates senescence (Thimann et al. 1974) and is a limiting factor in the synthesis of glutathione, a thiol implicated in resistance to biotic and abiotic stresses (May et al. 1998). Cysteine synthase was detected as induced by water deficit in maize (Riccardi et al. 1998) and by Fe-deficiency in barley roots (Negishi et al. 2002). Hemoglobins are most commonly recognized for their ability to act as O_2 carriers, however they are also well known regulators of NO homeostasis, protecting against nitrosative stress and modulating the signalling functions of NO (reviewed in Perazzolli et al. 2006). Nitric oxide (NO) plays a crucial role in the modulation of several plant physiological processes, including disease resistance, growth, development and abiotic stress responses such as salinity and osmotic stress, high temperature, UV radiation stress and anoxia. Therefore, the wide variety of sources of NO and its effects (free NO is highly reactive) suggests the necessity of detoxification mechanisms to control its level, reactivity and signalling functions. The up-regulation of a hemoglobin gene by Fe-deficiency may be related to its role in NO homeostasis.

Expression analysis of individual transcripts

The expression pattern of some selected clones in rice shoots under Fe-deficiency and Fe-sufficient conditions was further evaluated by semi-quantitative RT-PCR, using an actin gene as a control for equal loading. To confirm the up-regulated expression of the isolated genes, first-strand cDNA from 3, 6 and 9 days of exposure to Fe-deficiency or Fe-sufficient condition was used. As seen in Fig. 3, the results obtained confirm the differences in expression found by the RDA methodology. Interestingly, several tested genes (*RAC*, *Bet3*, *Alb3*, *Dynamin*, *TPI*, *HEXBP*, *PCS*, *SAMS* e *ADF5*) showed the same pattern of expression, with a decrease after 6 and 9 days in the control treatment and an increase at the same periods for Fe-deficiency. The lower expression seen in the control treatment after 6 and 9 days compared to 3 days is probably due to the change in Fe source when the plants were transferred from the adaptation solution (3.2 ppm Fe as $FeCl_3.6H_2O$) to the control solution (6.5 ppm Fe as $FeSO_4.7H_2O$). The lower amounts of available iron in the adaptation solution could be inducing mild Fe-deficiency responses in the plants. Young rice plants are known to be highly susceptible to Fe-deficiency, due to low production of phytosiderophores (Mori et al. 1991). Transfer to the control solution, with more available iron, would have resulted in decreased expression of such genes.

Fig. 3

Final considerations

Using RDA technique, we were able to detect 32 sequences activated by Fe-deficiency in rice shoots. Further analysis, in roots, may reveal other responsive genes. From the genes we identified in shoots, 28 had not been previously related to Fe-deficiency responses in plants. *TPI*, *ADF5*, Cysteine synthase and *SAMS* had already been correlated with this type of abiotic stress. The analysis of sequences suggests that a

significant amount of genes (about 30%) have overlapping functions in abiotic and even biotic stresses, not being restricted to iron stress, but rather should be considered in the context of a general stress response. Analysis of the sequences isolated also suggests that Fe-deficiency, as well as other biotic and abiotic stresses, can trigger leaf senescence. In general, senescence seems to have a biological function. For example, flower petals senesce after fertilization, and whole plant populations senesce after seed bearing. This suggests that the initiation of senescence is genetically predetermined, in a fine-tuned complex regulatory network. It is a highly regulated, ordered series of events involving the decline of photosynthetic activity, disintegration of chloroplasts, breakdown of biomolecules, loss of chlorophyll and the recycling of valuable nutrients to other parts of the plant. However, senescence can be prematurely activated when plants face unfavorable habitats. The other isolated sequences, with no correlation with Fe-deficiency or linked to other stresses, will need further analysis to have their functions in the Fe-deficiency response elucidated. It is 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 useful starting points for more in depth analyses of the molecular mechanisms behind Fe-deficiency stress in rice.

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Table 1. Gene-specific PCR primers used for semi-quantitative RT-PCR

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'
RAC	CATAGGAGGAGGAGGGCAAG	CGGCCTGCTTAAAAGGTGTA
Bet3	CACATGCTGCCTGTTTGTTC	CTACTTGATGCTGCCGTGAA
CEO	CGGATGGTGAGAGAAGCAA	CGGAAACCGATGACAGAAAT
Hemoglobin	CATGGACGATGGAATTCAGA	TCCTCCAGAGGCAGGTTCTA
Alb3	GGCACACAGCAAGATGAAAA	TTTGGTACCCACATTGCAGA
SucSynth	CAGACTCGGATGCAAAGGAT	TAAATTGAGCTGCGATGTGG
Dynamin	CCGCCAACATCATATTTTCA	TCTATTCTTCGAGCCCATGC
TPI	GCGTGGGAAGATGTCTGAAG	GGAAGTGAAGGGGAGGAAAA
HEXBP	TCACCAAACACAAGGAAGCA	TTGCAGGCACAAGTGATGAT
RWC3	ATCAGGGCAATTCCATTCAA	AGGAACGTCGACTCCACAAT
PCS	CGTTGCTCCGATTCTTTCAT	AATTGCTGCCAGATTCTGCT
SAMS	GATGGATGGAATTGGTTCGT	AACATGCCTCACTCGGAAAC
ADF5	ACGAGGGTTTGTGAATTTGG	TTTCTACGTCACCGACATGG
β-Gluc	GCCTACTGGTTCAGGGACAT	TTCATAGCCAGCTCTGATGC

Table 2. Up-regulated genes in rice shoots under Fe-deficiency

Functional Categories	Rice Pipeline clone No.	Description	Redundancy (%)	*E*-value
Photosynthesis				
	J013001C24	Ribulose-1,5-biphosphate carboxylase activase	174 (73%)	0.0
	J013131K08	Chloroplast membrane protein ALBINO3	2 (0,8%)	0.0
Carbohydrate metabolism				
	J013129F05	Glycine decarboxylase (glycine cleavage system P-protein)	8 (3,3%)	1e-119
	J033117C01	Chloroplast β-glucosidase – Glycosil hydrolase	5 (2,1%)	1e-165
	001-113-F06	Putative sucrose synthase, UDP-sucrose glucosyltransferase	2 (0,8%)	5e-49
	001-035-H02	Triose-phosphate isomerase (TPI)	2 (0,8%)	1e-165
	J013066N17	NADH-ubiquinone oxidoreductase complex I, 21 kDa subunit	2 (0,8%)	2e-23
Metal-binding				
	J013027D03	COBRA-like protein 7, Phytochelatin synthase-related	1 (0,4%)	1e-108
Protein degradation				
	J013116J09	UBC2 (ubiquitin-conjugating enzyme E2)	2 (0,8%)	2e-26
Lipid metabolism				
	J033093M11	GDSL-motif lipase	1 (0,4%)	9e-95
Structural				
	J033122A14	Putative Bet3 - transport protein particle (TRAPP) component	4 (1,7%)	0.0
	J013074K16	Dynamin-like protein	2 (0,8%)	4e-78
	001-002-G04	Centromere protein-like, kinase interacting	1 (0,4%)	1e-164
	J013170K24	ADF5 (Actin-depolymerizing factor 5)	1 (0,4%)	1e-111
Protein-protein interaction				
	J023139M21	WWE domain-containing CEO protein	3 (1,3%)	5e-65
	002-150-B07	Chloroplast heat shock protein-binding protein	1 (0,4%)	1e-107
Transport				
	J013002E02	Water channel-like protein RWC3 (MIP family)	1 (0,4%)	0.0
Nucleotide-binding protein				
	001-020-G10	Hexamer-binding protein family (HEXBP) – Zinc finger protein	2 (0,8%)	1e-160
	J023141I23	SF2/ASF-like pre-mRNA splicing factor	1 (0,4%)	3e-44
Stress response				
	J013061O18	Putative negative light-regulated protein	4 (1,7%)	1e-148
	J023123M01	HECT and RCC1-like domain UVB-resistance protein	4 (1,7%)	1e-115
	J023082J11	Phenylalanine ammonia-lyase	1 (0,4%)	3e-35
	J033107J07	Heat shock protein 70 kDa (HSP70)	1 (0,4%)	1e-53
Signal transduction				
	J013001C20	Signal transducer of phototropic response (RPT2)	2 (0,8%)	8e-86
Protein biosynthesis				
	J023054I18	Putative 60S ribosomal protein L13	3 (1,3%)	1e-100
Protein modification				
	J013135O21	Putative N-terminal acetyltransferase	1 (0,4%)	2e-96
Others				
	J033075H07	Truncated 2-on-2 hemoglobin	2 (0,8%)	1e-150
	J023084C02	Cysteine synthase	2 (0,8%)	3e-85
	002-134-E10	S-adenosylmethionine synthetase (SAMS)	1 (0,4%)	2e-87
	J023115F20	Putative deoxycytidine/cytidine deaminase protein – zinc ion-binding	1 (0,4%)	8e-73
Unknown				
	J033031K13	Unknown protein	1 (0,4%)	0.0
	J013127F10	Unknown protein	1 (0,4%)	9e-89

Figure legends

Figure 1. Differential products 1 and 2 obtained by RDA experiments. Fe-deficient rice shoots cDNAs were subtracted from control condition rice shoots cDNAs by representational difference analysis after the first (DP1) and second (DP2) rounds of hybridization. DP1 and DP2 were fractionated on a 1,2% agarose gel electrophoresis and stained with ethidium bromide. **M.** 100 bp DNA Ladder; **1.** Driver control condition; **2.** Tester Fe- condition; **3.** Differential Product 1 (1:50); **4.** Differential Product 2 (1:500).

Figure 2. Total soluble sugars content from leaves submitted to control (white bars) and Fe-deficient (black bars) conditions for 3, 6 and 9 days. Means with different letters are different by the Duncan test ($P \leq 0,05$). DW = Dry Weight.

Figure 3. Semi-quantitative RT-PCR expression analysis of selected genes identified by RDA. Band intensity of each PCR product was normalized in relation to the optical density obtained for the actin gene. Numbers below each lane represent a percentage in relation to the highest normalized optical density. Numbers in parentheses indicate the number of cycles needed to detect the PCR product. Control, control condition; -Fe, Fe-deficiency condition; *RAC*, Rubisco activase; *Alb3*, Albino3; *SucSynth*, Sucrose synthase; *TPI*, Triose-phosphate isomerase; *HEXBP*, Hexamer-binding protein; *RWC3*, Water channel protein; *PCS*, Phytochelatin synthase; *SAMS*, S-adenosylmethionine synthetase; *ADF5*, Actin-depolymerizing factor 5; *β-Gluc*, beta-Glucosidase.

Figure 1

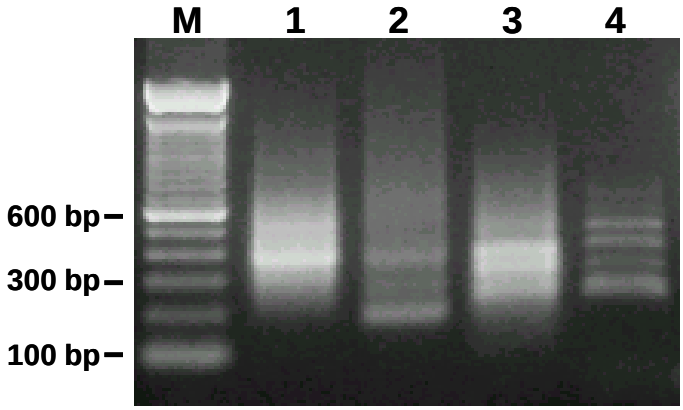


Figure 2

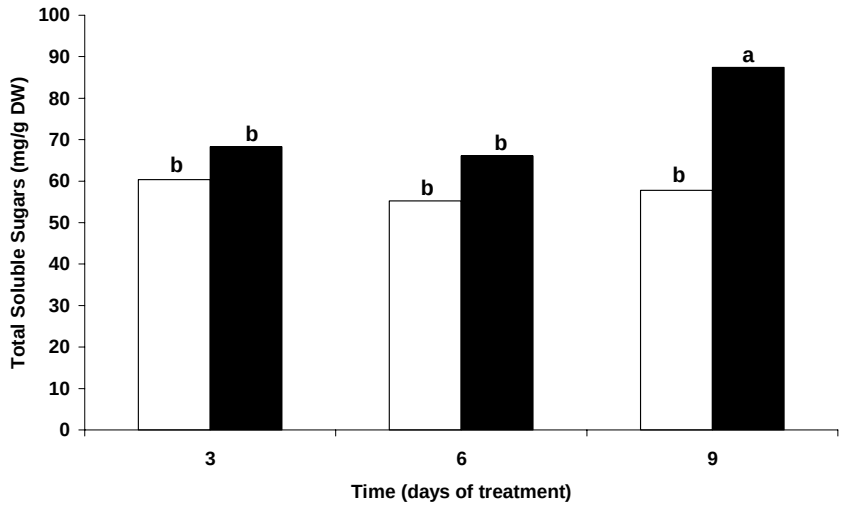
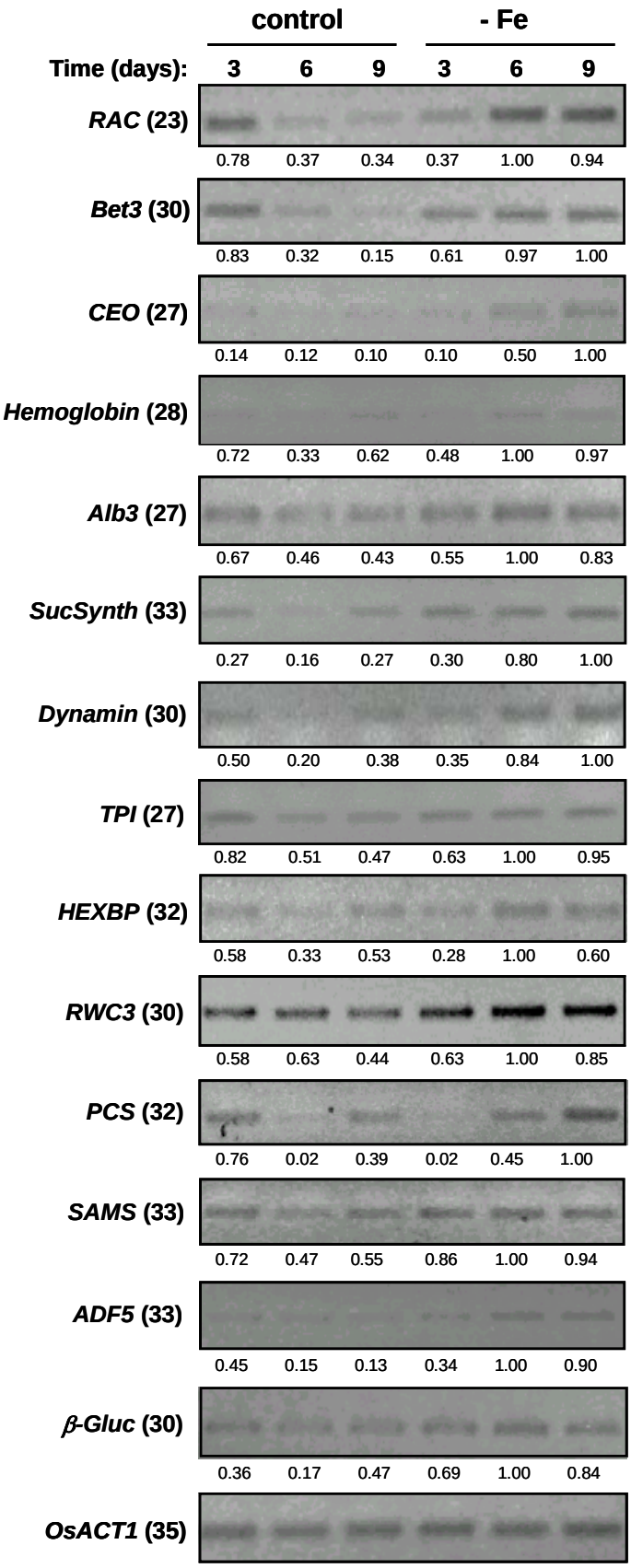

Total Soluble Sugars (mg/g DW)
100
90
80
70
60
50
40
30
20
10
0
b
b
b
b
b
a
3
6
9
Time (days of treatment)

Figure 3


control
- Fe
Time (days): 3 6 9 3 6 9
RAC (23)
0.78 0.37 0.34 0.37 1.00 0.94
Bet3 (30)
0.83 0.32 0.15 0.61 0.97 1.00
CEO (27)
0.14 0.12 0.10 0.10 0.50 1.00
Hemoglobin (28)
0.72 0.33 0.62 0.48 1.00 0.97
Alb3 (27)
0.67 0.46 0.43 0.55 1.00 0.83
SucSynth (33)
0.27 0.16 0.27 0.30 0.80 1.00
Dynamin (30)
0.50 0.20 0.38 0.35 0.84 1.00
TPI (27)
0.82 0.51 0.47 0.63 1.00 0.95
HEXBP (32)
0.58 0.33 0.53 0.28 1.00 0.60
RWC3 (30)
0.58 0.63 0.44 0.63 1.00 0.85
PCS (32)
0.76 0.02 0.39 0.02 0.45 1.00
SAMS (33)
0.72 0.47 0.55 0.86 1.00 0.94
ADF5 (33)
0.45 0.15 0.13 0.34 1.00 0.90
β-Gluc (30)
0.36 0.17 0.47 0.69 1.00 0.84
OsACT1 (35)

CONCLUSÕES

A deficiência de ferro é considerada um dos estresses abióticos que mais diminui a produtividade em lavouras de arroz. Com o intuito de identificar genes cuja expressão é induzida em partes aéreas de plantas de arroz submetidas a baixos suprimentos de ferro, utilizamos a técnica de cDNA-RDA (*Representational Difference Analysis*), que baseia-se em passos sucessivos de hibridização de duas populações contrastantes de cDNA seguida de PCR com *primers* específicos para somente uma das populações testadas.

O uso desta técnica mostrou-se bastante plausível no estudo de partes aéreas de arroz, permitindo a identificação de trinta e duas seqüências cuja expressão é aumentada após 3, 6 e 9 dias de exposição à deficiência de ferro. Para verificar os diferentes níveis de expressão foram realizados experimentos de RT-PCR semi-quantitativo, que mostraram ser uma alternativa rápida e simples para validar a biblioteca de cDNA gerada.

Através da análise das seqüências isoladas, foi possível separá-las em diversas categorias funcionais. Muitas das seqüências isoladas são descritas na literatura como participantes no processo de resposta de diversas espécies de plantas a outros estresses abióticos e bióticos, principalmente seca e altas temperaturas, sugerindo que são genes de resposta geral a estresse, não sendo restritos a estresse por deficiência de ferro. Porém, essa é a primeira em vez que são descritos como participantes na resposta a baixas concentrações de ferro.

Também foram isoladas seqüências que são tipicamente encontradas em folhas que estão sofrendo processo de senescência, como lipase, cisteína sintase e uma enzima que se conjuga a ubiquitina. Além dessas, também foram encontradas cinco seqüências envolvidas no metabolismo de carboidratos, sugerindo que as plantas submetidas à deficiência de ferro apresentam maior conteúdo de açúcares do que as plantas submetidas ao tratamento controle. Tal fato foi confirmado através da análise do conteúdo de açúcares solúveis totais presentes nas amostras de folhas submetidas aos dois tratamentos, em que foi verificado um acúmulo de açúcares nas folhas expostas à deficiência de ferro por nove dias. A literatura sugere que o acúmulo de açúcares provavelmente seja um dos sinais que induzem a senescência em folhas de diversas espécies de plantas. Dessa forma, a deficiência de ferro, assim como outros fatores

desfavoráveis para as plantas, pode levar a um processo de senescência, provavelmente induzido por acúmulo de carboidratos.

Dos 32 genes encontrados como ativados por deficiência de ferro, dois deles são fortes candidatos para investigações futuras em relação à resposta à deficiência de ferro, *CEO* e *HEXBP*. O primeiro é um cofator de fatores de transcrição envolvidos com resposta a estresses bióticos e abióticos e poderia participar da ativação de genes classicamente envolvidos na absorção de ferro em condições de baixo suprimento deste nutriente. O segundo é um fator de transcrição e também poderia estar envolvido com a resposta de plantas de arroz à deficiência de ferro. Análises fenotípicas e moleculares de plantas de arroz mutantes para esses genes seriam extremamente úteis para verificar a correlação de suas expressões com os níveis de adaptabilidade à deficiência de ferro.

Um maior número de seqüências responsivas diretamente a estresse por deficiência de ferro possivelmente sejam encontradas nas raízes, que serão investigadas em experimentos futuros.