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Mecanismos moleculares de resposta ao estresse por excesso de ferro em arroz

Artur Teixeira de Araujo Junior

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Orientador: Antonio Costa de Oliveira (UFPel)
Coorientador: Luciano Carlos Maia (UFPel)

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BANCA EXAMINADORA

Prof. Dr. Antonio Costa de Oliveira (Orientador, UFPel, Departamento de Fitotecnia)

Prof. Dr. Luciano Carlos Maia (Coorientador, UFPel, Departamento de Fitotecnia)

Prof.ª Dr.ª Camila Pegoraro (UFPel, Departamento de Fitotecnia)

Prof. Dr. Cesar Valmor Rombaldi (UFPel, Departamento de Ciência e Tecnologia Agroindustrial)

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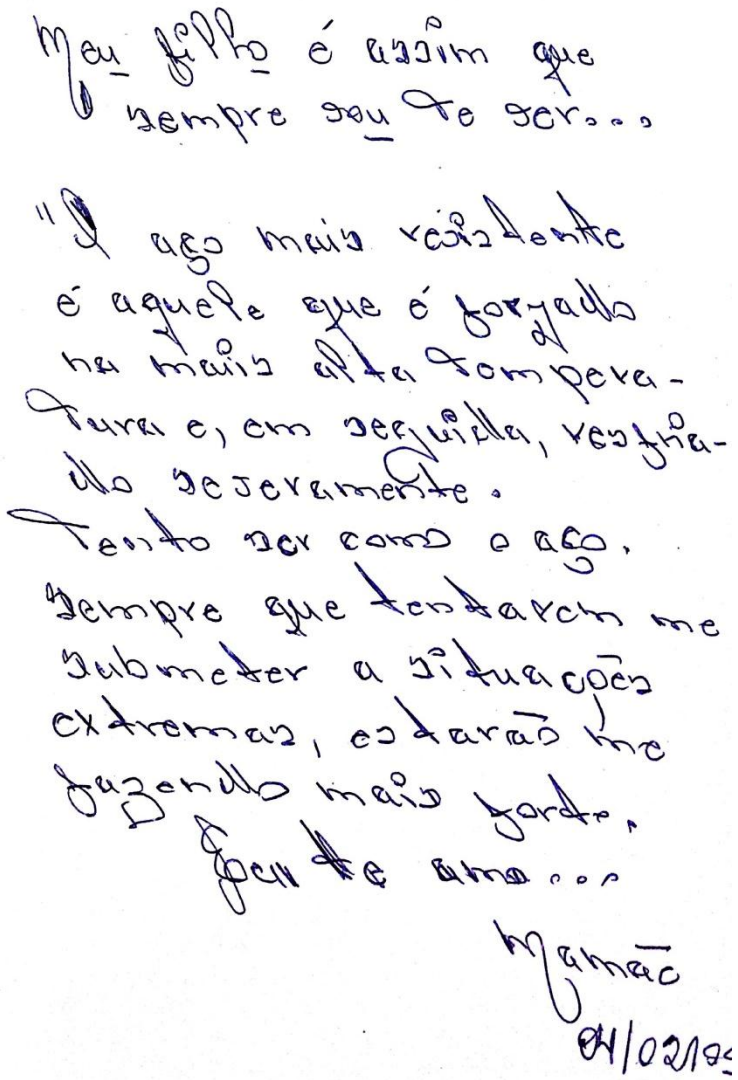
Meu filho é assim que
sempre vou te ver...

"O aço mais resistente
é aquele que é forjado
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Tente ser como o aço.
Sempre que tentarem me
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Eu te amo...
Mamãe
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04/02/2005”

Dulcinea Gonçalves Teixeira

Resumo

Araujo Junior, Artur Teixeira de. **Mecanismos moleculares de resposta ao estresse por excesso de ferro em arroz.** 2016. 167f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

O ferro (Fe) é um nutriente essencial para o crescimento e desenvolvimento das plantas. Contudo, a disponibilidade deste elemento varia devido às condições do solo e sistema de cultivo. Tanto a absorção excessiva quanto a deficiência deste elemento pode afetar as plantas ocasionando a perda da produtividade. No Rio Grande do Sul, problemas provenientes do excesso de ferro já foram relatados, sendo que o uso de cultivares tolerantes torna-se uma importante estratégia para solucionar este problema. Desta forma, quanto mais informações a respeito dos mecanismos que conferem essa tolerância nas plantas forem adquiridos, mais fácil fica sua aplicação nos programas de melhoramento. O objetivo desta dissertação foi estudar o transcriptoma de uma cultivar brasileira de arroz caracterizada como tolerante ao excesso de ferro (BRS Querência). Para isso, as plantas foram submetida à condição de estresse (300 mg L^{-1} Fe^{+2}) no estádio V3 por 24 horas, o RNA das folhas foram extraídos e sequenciados pelo tecnologia de RNA-Seq. Tal estudo foi dividido nos seguintes capítulos: I) Lidando com o metabolismo de ferro no arroz: a partir do melhoramento visando a tolerância ao estresse até a biofortificação (Artigo de revisão submetido à revista *Genetics and Molecular Biology*); II) Perfil transcricional do amplo-genoma de arroz revela novos genes envolvidos na tolerância ao excesso de ferro (Artigo com provável submissão à revista *The Plant Genome*); III) Resposta ao estresse de plantas: o papel de *splicing* alternativo (Artigo com provável submissão à revista *Genome*); IV) A busca por arroz mais tolerante: como altas concentrações de ferro afetam o *splicing* alternativo? (Artigo aceito na revista *Transcriptomics: Open Access*). O estudo possibilitou a identificação de vários genes diferencialmente expressos, dentre esses pode-se destacar a ativação de genes que codificam para as proteínas responsáveis pela homeostase de ferro (transporte e armazenagem), para as proteínas de choque térmico e as proteínas do fotossistema II. Além disso, temos em destaque a repressão dos genes que codificam proteínas do metabolismo hormonal e de sinalização. A análise do *splicing* alternativo proporcionou a identificação da mudança do perfil de *splicing* na célula sobre a condição de estresse, tendo uma redução tanto da quantidade dos sítios de junção quanto do número de eventos. Os resultados obtidos representam um importante passo para o entendimento das estratégias de tolerância desempenhada por esta cultivar, auxiliando na identificação de possíveis genes alvos para serem utilizados pelos programas de melhoramento genético.

Palavras-chave: transcriptoma, toxicidade de ferro, estresse abiótico.

Abstract

Araujo Junior, Artur Teixeira de. **Molecular mechanisms of stress response under excess of iron in rice**. 2016. 167f. Dissertação (Mestrado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Iron (Fe) is an essential nutrient for the growth and development of plants. However, the availability of this element varies due to ground conditions and cropping system. Either the excessive absorption or the deficiency of this element may cause toxicity in plants leading to a loss of the productivity. In Rio Grande do Sul, problems from the excess of iron have been reported, and the use of tolerant cultivars becomes an important strategy to address this issue. Thus, the more information regarding the mechanisms that confer this tolerance in plants is known, the easier their application in breeding programs becomes. The aim of this work was to study the transcriptome of a Brazilian rice cultivar characterized as tolerant to excess of iron (BRS Querência). For this, the plants were submitted to stress conditions (300 mg L^{-1} Fe^{+2}) in the V3 stage for 24 hours, the RNA of the leaves was extracted and sequenced by RNA-Seq technology. Such a study was divided into the following chapters: I) Dealing with iron metabolism in rice: from breeding aiming at stress tolerance to biofortification (Review article submitted to the Genetics and Molecular Biology); II) Genome-wide transcriptional profile of rice reveals new genes involved in tolerance to iron (Article probable submission to the The Plant Genome); III) Stress response of plants: the role of alternative splicing (Review article probable submission to the Genome); IV) The search for more tolerant rice: how high concentrations of iron affect the alternative splicing? (Article accepted by Transcriptomics: Open Access). The study enabled the identification of several differentially expressed genes. Among these, we highlight the activation of genes encoding proteins responsible for iron homeostasis (transport and storage) the heat shock proteins and proteins of photosystem II. Furthermore, we have highlighted the repression of genes encoding the hormone metabolism and signaling proteins. The alternative splicing analysis provided the identification of a profile change in the cell on stress condition, having reduction in the quantity of the junction sites as well as in the number of events. The results represent an important step in the understanding of tolerance strategies performed by this cultivar, assisting in the identification of potential target genes for use by breeding programs.

Keywords: transcriptomics, iron toxicity, abiotic stress.

Lista de Figuras

Lista de Abreviaturas

3’UTRs – 3′ *untranslated regions* (regiões não traduzida na extremidade 3’)

5’UTRs – 5′ *untranslated regions* (regiões não traduzida na extremidade 5’)

CONAB – Companhia Nacional de Abastecimento

CDSs – *Coding Sequences* (sequências de codificação)

DDBJ – DNA *Data Bank of Japan* (Banco de Dados de DNA do Japão)

DNA – *Deoxyribonucleic acid* (ácido desoxirribonucleico)

DRA – DDBJ *Sequence Read Archive* (Arquivo de Sequências de Leitura)

EBI – *European Bioinformatics Institute* (Instituto Europeu de Bioinformática)

EMBL – *European Molecular Biology Laboratory* (Laboratório de Biologia Molecular Europeu)

Embrapa – Empresa Brasileira de Pesquisa Agropecuária

ENA – *European Nucleotide Archive* (Arquivo de Nucleotídeo Europeu)

FAO – *Food and Agriculture Organization* (Organização da agricultura e alimento)

Fe – Ferro

Fe(II) – Ferro na forma de íon férrico

FRD3 – *Ferric reductase defective 3* (Redutase férrica com defeito 3)

GO – *Gene Ontology* (Ontologia do gene)

HSPs – *Heat shock proteins* (Proteínas de choque térmico)

INSDC – *International Nucleotide Sequence Database Collaboration* (Colaboração Internacional de Banco de Dados de Nucleotídeo)

IRGSP – *International Rice Genome Sequencing Project* (Projeto Internacional de Sequenciamento do Genoma do Arroz)

L – Litros

Mb – Megabases

mg – Miligramas

MTN – *Methylthioadenosine nucleosidase* (Metiltioadenosina nucleosidase)

NCBI – *National Center for Biotechnology Information* (Centro Nacional de Informações sobre Biotecnologia)

PSII – *Photosystem* II (Fotossistema II)

RAP-DB – *Rice Annotation Project Database* (Bando de Dados de Projetos de Anotação de Arroz)

RNA – *Ribonucleic acid* (ácido ribonucleico)

RNA-Seq – RNA *sequencing* (sequenciamento de RNA)

ROS – *Reactive oxygen species* (Espécies reativas de oxigênio)

SA – *Splicing* Alternativo

SNG – Sequenciamento de Nova Geração (*Next-Generation Sequencing*)

SNPs – *Single Nucleotide Polymorphisms* (Polimorfismos de Nucleotídeo Único)

TAIR – *The Arabidopsis Information Resource* (Recurso de Informações de *Arabidopsis*)

V3 – Estádio vegetativo no terceiro nó

VIT1 – *Vacuolar Iron Transporter* 1 (Transportador de ferro vacuolar 1)

Sumário

1 INTRODUÇÃO GERAL

No decorrer dos últimos anos, os avanços na bioinformática e na biologia molecular vêm promovendo o aparecimento de novas tecnologias. Dentre essas tecnologias temos o aprimoramento e o surgimento de novas técnicas de análise, no qual, possuem um amplo potencial de aplicação, incluindo, por exemplo, a análise de expressão gênica, a predição de estrutura proteica, a interação proteína-proteína, entre outros, promovendo assim, grandes descobertas a respeito de vários mecanismos que os organismos realizam (Masulli & Tagliaferri, 2010).

Entre os fatores que prejudicam a produtividade e a qualidade da produção agrícola temos a presença dos estresses bióticos e abióticos, sendo considerado um dos grandes responsáveis por essa diminuição. Desta forma, tem ocorrido um empenho no mundo científico para elucidar os mecanismos envolvidos na resposta de tolerância das plantas, possibilitando a identificação de importantes genes responsivos às condições adversas.

Dentre as tecnologias desenvolvidas que auxiliam na identificação destes mecanismos de tolerância temos o RNA *sequencing* (RNA-Seq), capaz de caracterizar todo o transcriptoma e quantificar os diferentes níveis de expressão gênica provenientes de distintos tecidos do organismo que se deseja analisar (Levin et al., 2010).

Com os resultados obtidos dessa tecnologia pode-se analisar mais facilmente a taxa de *splicing* alternativo (SA), fenômeno que está envolvido em muitos processos fisiológicos da planta, inclusive nas respostas aos diversos estresses bióticos e abióticos (Barbazuk et al., 2008; Reddy, 2001). Sendo considerado um mecanismo de regulação de expressão da célula, podendo promover uma maior plasticidade e versatilidade no genoma (Staiger & Brown, 2013). Deste modo, entender as mudanças no perfil de SA pode contribuir para uma maior compreensão da resposta da planta ao estresse.

O ferro (Fe) é um importante micronutriente para grande parte dos organismos, participando de vários processos metabólicos (Rout & Sahoo, 2015). Contudo, tanto sua deficiência quanto o seu excesso podem acarretar em condições de estresse (Connolly & Guerinot, 2002). Elevados níveis de Fe na forma de íon férrico Fe(II) no solo podem promover toxidez nas plantas (Mongon et al., 2014), podendo afetar seu desenvolvimento e crescimento, ocasionando em grandes

perdas na safra (Sahrawat, 2004; Zhang et al., 2014). Nos solos alagados existe uma maior probabilidade de se encontrar elevados níveis de Fe(II) (Mongon et al., 2014).

Uma das culturas afetadas por este estresse é o arroz (*Oryza sativa* L.), principalmente os de ecossistema de cultivo irrigado. O arroz é considerado uma das espécies mais importantes para a sociedade, tendo uma grande relevância no setor social, econômico e científico (Yu et al., 2002; Finatto et al., 2015). O Brasil é o nono maior produtor mundial e coloca-se como o maior produtor fora do continente asiático. O estado do Rio Grande do Sul é o principal produtor, sendo responsável por mais de dois terços da produção nacional (CONAB, 2016). Contudo, estima-se que no Rio Grande do Sul, em locais onde a toxidez por ferro é acentuada, a perda na produção pode chegar a 20%, sendo os casos mais extremos relatados em localidades da África, com perdas de até 100% (Sahrawat, 2005).

A intensidade do efeito do estresse na planta depende de vários componentes (tipo da cultivar, época de plantio, manejo da água, entre outros) (Sahrawat, 2005). Para minimizar os efeitos, tem se adotado a utilização de genótipos resistentes, sendo uma forma mais econômica e eficiente para contornar o problema (Sahrawat et al., 1996). A cultivar BRS Querência é caracterizada por possuir bons níveis de tolerâncias aos estresses abióticos (tolerância ao Fe, tolerante ao frio nos estádios iniciais e moderadamente tolerante ao estresse salino), possuindo também uma elevada produtividade, se tornando assim, uma cultivar interessante para analisar as estratégias de tolerância (Benitez et al., 2011).

A compreensão dos mecanismos de homeostase do ferro em plantas possui uma elevada significância tanto do ponto de vista agronômico quanto científico. Este conhecimento auxiliaria na identificação de importantes genes que contribuem para a sobrevivência da planta em condições adversas, possibilitando o desenvolvimento de plantas mais tolerantes e de alimentos biofortificados com Fe (Gura, 1999; Grotz & Guerinot, 2002).

2 REVISÃO BIBLIOGRÁFICA

2.1 A cultura do arroz

Certas características da produção, consumo e constituição genética torna o arroz uma das culturas mais importantes do mundo (Nayar, 2014). O arroz é cultivado em aproximadamente 177 países do mundo, estando presente desde latitudes que vão de 53° Norte a 35° Sul (Lu & Chang, 1980).

O gênero *Oryza* provavelmente teve sua origem por volta de pelo menos 130 milhões de anos atrás (Chang, 1976), tendo como centro de origem na China (Bautista et al., 2001). Este gênero é composto de duas espécies domesticadas (*Oryza sativa* L. e *Oryza glaberrima* Steud.) e 22 espécies selvagens (Vaughan, 1994), possuindo uma ampla gama de características morfológicas (altura, perfilhamento, floração, hábito de crescimento, panícula, folha, entre outras) (Figura 1), além de diferentes níveis de adaptação aos habitats (Ali et al., 2010).

Figura 1. Fotografia demonstrando 12 espécies do gênero *Oryza* todas no mesmo estádio de desenvolvimento. Adaptado de Zhang et al. (2013).

Apesar das espécies selvagens serem consideradas reservatórios de muitos genes importantes, principalmente genes associados à resistência a estresses bióticos e abióticos, elas possuem um conjunto de características agronômicas não desejáveis, como por exemplo, baixa produção de sementes, quebra de grãos, entre

outras. Assim, a transferência de genes úteis de espécies selvagens para espécies cultivadas é dificultada (Brar & Khush, 1986; Brar & Khush, 1997). Desta forma, encontrar genes importantes dentro das espécies domesticadas se torna uma boa alternativa para os programas de melhoramento genético.

A espécie *O. sativa* é amplamente cultivada em regiões tropicais e temperadas, sendo dividida em três outras subespécies (*indica, japonica e javanica*), enquanto que a *O. glaberrima* é endêmica do leste da África possuindo duas subespécies (*nivara* e *rufipogon*) (Vaughan et al., 2003; Londo et al., 2006).

No Brasil, o arroz foi introduzido no início do século 16 pelos portugueses se tornando ao passar do tempo um dos principais alimentos de consumo interno (Mantovani et al., 2008).

2.2 Importância do arroz

O arroz é considerado uma fonte primária de alimento para a metade da população do mundo. Sua importância na dieta varia entre os países. Contudo, aproximadamente um quarto das calorias consumidas por toda a população mundial provém do arroz. Sendo que em alguns países pode chegar a 70% da ingestão de calorias diárias (Kole, 2014).

Em algumas regiões, temos a grande presença do arroz no meio social, sendo utilizado nas práticas religiosas e nas tradições culturais (Carney & Rosomoff, 2009). O arroz é considerado uma espécie modelo dentro da família Poaceae, tendo um elevado número de estudos em comparação com outros cereais (Figura 2). Isso ocorre devido a uma série de características, entre elas temos o tamanho do seu genoma (389 Mb), classificado como pequeno quando comparado com outros cereais de importância econômica (milho - 2.500 Mb, cevada - 4.900 Mb e trigo - 16.000 Mb). Além disso, o arroz possui uma alta sintenia e colinearidade com os demais cereais, sendo utilizado para o entendimento da estrutura e função do genoma (Zhang et al., 2013). Outra característica é em relação ao completo sequenciamento do genoma da subespécie *japonica* cultivar Nipponbare em 2005, proveniente do Projeto Internacional de Sequenciamento do Genoma do Arroz (IRGSP- *International Rice Genome Sequencing Project*) (Bennetzen & Freeling,

1993; Devos, 2005). Promovendo assim, uma grande evolução nos estudos de arroz voltados para as análises moleculares e genéticas (Tyagi et al., 2004).

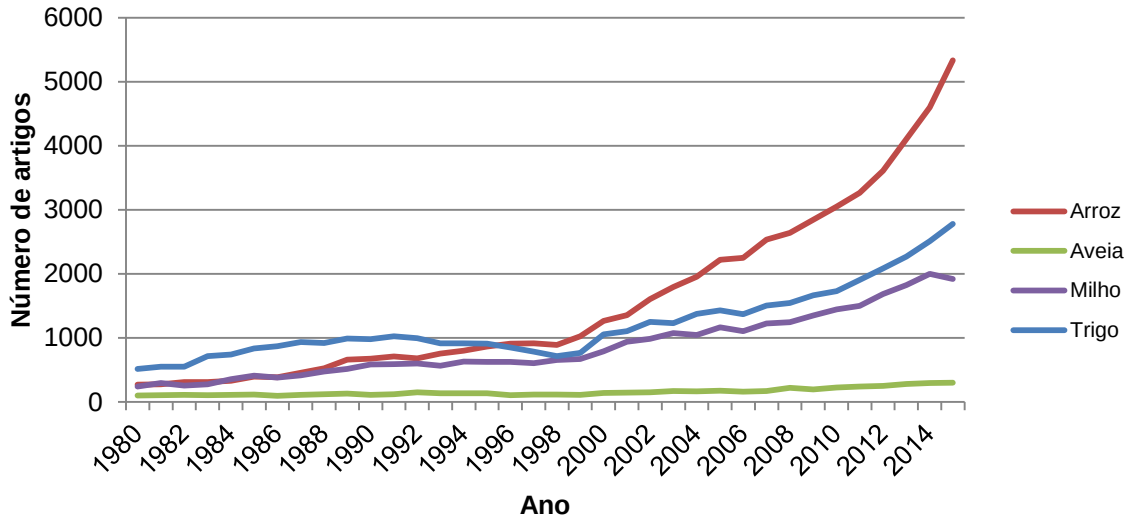

Figura 2. Evolução da quantidade de publicações ao longo do tempo dos principais cereais. Número de artigos encontrados no banco de dados do PubMed (http://www.ncbi.nlm.nih.gov/pubmed) utilizando as palavras '*rice'*, '*oat',* '*maize'* e '*wheat'*.

Considerando sua importância em relação ao cultivo, temos que o arroz ocupa a posição de segundo cereal mais cultivado no mundo. Sua produção mundial foi de aproximadamente 740 milhões de toneladas em 2015, ocupando uma área de plantio de 161 milhões de hectares por ano. Contudo, comparando os dados obtidos em 2014, nota-se uma diminuição tanto na produção quanto na área plantada (Figura 3A), o principal motivo dessa redução foi devido às condições climáticas desfavoráveis em 2015, tendo a ocorrência de um forte *El Niño* (FAO, 2015).

Grande parte do cultivo e consumo decorre do continente Asiático, tendo como destaque a Índia que possui a maior área cultivada de arroz (45 milhões de hectares) e a China que é o maior produtor de arroz (207,5 milhões de toneladas). O Brasil também ocupa uma posição de destaque sendo o nono maior produtor mundial e o primeiro país fora da Ásia em produção e consumo, produzindo em torno de 12,4 milhões de toneladas (Figura 3B). O Rio Grande do Sul tem uma relevância fundamental nessa produção, sendo responsável por mais de dois terços da produção de arroz do Brasil. Obtendo rendimentos de até 7,7 toneladas por hectare (FAO, 2015).

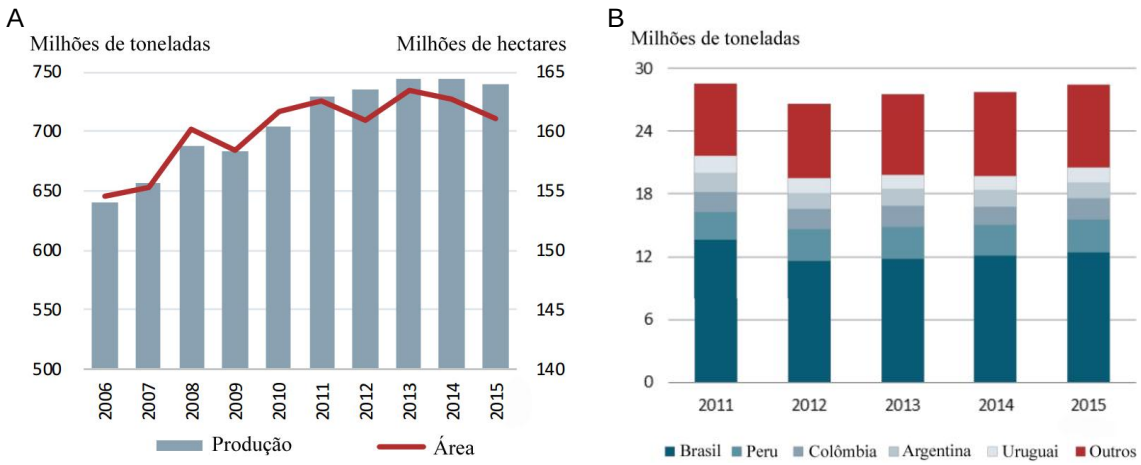

Figura 3. Panorama da cultura do arroz ao longo dos anos. **(A)** Evolução global da produção e da área plantada de arroz de 2006 a 2015. **(B)** Evolução da produção de arroz na América Latina e Caribe de 2011 a 2015. Adaptado de FAO (2015).

O uso de cultivares modernas proporcionou ao estado do Rio Grande do Sul um aumento no rendimento da produção de aproximadamente 35%. Visando esta melhoria, teve-se uma crescente busca para desenvolver novas cultivares que possibilitariam uma maior produtividade, um aumento na resistência aos principais estresses ambientais e um menor uso de insumos agrícolas. Das cultivares modernas lançadas, destaca-se a BRS Querência, pertencente à espécie *O. sativa* ssp. *indica*, sendo lançada pela Empresa Brasileira de Pesquisa Agropecuária (Embrapa) em 2004, derivada de um cruzamento controlado entre a linhagem CL 246 e a cultivar Zho Fee N° 10 (Fagundes et al., 2005). Uma das suas principais características provém de sua reação aos estresses abióticos, caracterizada como possuindo um bom nível tolerância ao Fe, tolerante ao frio nos estádios iniciais e moderadamente tolerante ao estresse salino (Benitez et al., 2011). Tornando-se uma cultivar importante para se estudar as estratégias de tolerância, identificando genes diferencialmente expressos e analisando os principais processos metabólicos que são afetados na resposta ao estresse.

2.3 Toxidez de ferro em arroz

Nos estados do Rio Grande do Sul e Santa Catarina, temos o cultivo de arroz irrigado, encontrado, principalmente, nos ecossistemas de terras baixas. Uma das características que este ecossistema apresenta é a deficiência de drenagem,

acarretando na inundação do solo e podendo promover uma elevação dos teores de Fe(II), que pode chegar a ser tóxico para as plantas (Becana et al., 1998; Embrapa, 2005).

A toxidez por excesso de ferro é um importante estresse abiótico, limitando a produção do arroz irrigado de vários países, podendo ter perdas de até 100% da produção (Dobermann & Fairhurst, 1977). Sob condições adversas a planta desenvolve um mecanismo resposta, realizando uma expressão diferenciada de uma série de genes cujos produtos são responsáveis por promover uma resposta de tolerância ou sensibilidade ao estresse. Desta forma, realizar estudos que visam à identificação de genes que estão envolvidos na tolerância da planta ao excesso de ferro auxiliaria nos programas de melhoramento genéticos a desenvolver novas cultivares com esta característica.

2.4 Uso da bioinformática no melhoramento genético

Para realizar um melhor estudo dos vários elementos biológicos mensuráveis teve-se a formação de um modelo conceitual denominado *'omic space'*, no qual, é subdividido em várias camadas, incluindo transcritômica, proteômica, metabolômica, entre outros (Toyoda & Wada, 2004). Os estudos em cada área de investigação podem gerar um grande conjunto de dados, sendo necessário um recurso que ajude no manuseo de toda a informação. Com isso, a bioinformática foi se tornando cada vez mais importante, auxiliando na mineração de dados e vinculando esse conhecimento com o seu significado biológico (Mochida et al., 2010).

A bioinformática pode ser definida como o estudo da informação existente nos seres vivos, sendo tanto uma ferramenta operacional, quanto a aplicação direta da Tecnologia da Informação no manejo de dados, desenvolvendo ferramentas computacionais responsáveis por adquirir, armazenar, organizar, analisar e visualizar os dados biológicos (Gibas & Jambeck, 2002).

Um dos conjuntos de dados gerados provém do sequenciamento, no qual, são armazenados e distribuídos pelo *International Nucleotide Sequence Database Collaboration* (INSDC) que consiste no NCBI *Sequence Read Archive* (Wheeler et al., 2007), no *European Nucleotide Archive* (ENA) proveniente do EMBL-EBI (Leinonen et al., 2011) e no DDBJ *Sequence Read Archive* (DRA) (Kodama et al.,

2012). Sendo que os dados de sequências de DNA genômico utilizados como genomas de referência ficam disponíveis em bancos de dados da *web*, tais como: *The Arabidopsis Information Resource* (TAIR) para *Arabidopsis* (Lamesch et al., 2012) e *Rice Annotation Project Database* (RAP-DB) para o arroz (Sakai et al., 2013).

Os recursos de bioinformática juntamente com as bases de dados da *web* exercem uma grande relevância para a utilização mais eficaz dos dados de pesquisa, sendo consideradas importantes estratégias para identificar os sistemas moleculares e as propriedades biológicas de diferentes espécies, além de acelerar a descoberta de genes e análises funcionais, contribuindo significativamente para o uso desta informação nos programas de melhoramento de plantas (Hakeem et al., 2013).

As plantas são organismos sésseis e sob condições ambientais adversas precisam adaptar os seus processos biológicos a essas novas condições, desenvolvendo diferentes mecanismos de respostas (Kalisz & Kramer, 2008). Essas respostas ambientais desencadeiam uma expressão diferenciada dos genes (Bossdorf et al., 2007; Sani et al., 2013). Entre as abordagens de identificação desta alteração tem sido utilizado o sequenciamento de nova geração (SNG). Nos últimos anos, tem-se observado um aumento na utilização desta tecnologia, ocorrendo também, uma diminuição de seus custos. A análise da expressão através do SNG é realizada em vários passos, tendo o grande uso da bioinformática na manipulação desses dados (Sablok et al., 2015).

O arroz foi uma das primeiras culturas a ter uma sequência de genoma de referência e foi utilizado como uma espécie modelo para o uso de SNG. O resequenciamento do arroz permitiu a identificação de genes domesticados presentes em áreas de baixa diversidade do genoma, provenientes de um possível resultado da seleção humana. Além disso, possibilitou observar que o arroz *japonica* e *indica* compartilham áreas comuns de baixa diversidade, possivelmente devido a introgressão de uma população para outra após a seleção (Jha et al., 2015). Através das informações presentes nas sequências do genoma foi possível auxiliar na compreensão da estrutura do genoma do arroz e suas evoluções, bem como descobrir novos genes, incluindo genes associados às características complexas de elevada importância na agricultura.

2.5 Uso da tecnologia de RNA-Seq

O sequenciamento dos diferentes níveis de expressão do RNA através da tecnologia do RNA-Seq vem crescendo e se popularizando rapidamente. Isso ocorre principalmente devido a redução do custo no sequenciamento, o aumento da confiabilidade e na reprodutibilidade dos resultados, e na detecção de transcritos de organismos que não possuem um genoma ou um transcriptoma de referência (Liu et al., 2012). Além de quantificar os níveis de expressão gênica o RNA-Seq possibilita a identificação de diferenças presentes nas regiões 3'UTRs/5'UTRs, nas regiões de *splicing*, nos SNPs e nos CDSs (Trapnell et al., 2011). Desta forma, a utilização dessa tecnologia para fins de entender a resposta da BRS Querência nas condições de estresse por excesso de ferro se torna uma estratégia interessante e viável, podendo auxiliar no descobrimento e na identificação de um possível mecanismo de tolerância.

3 HIPÓTESE E OBJETIVOS

3.1 Hipótese

O estresse por excesso de ferro pode provocar mudanças na expressão dos genes das folhas de arroz, demonstrando como a planta responde a este estresse.

A análise do perfil de expressão deve auxiliar na identificação de genes que codificam proteínas relacionadas com a homeostase de ferro.

3.2 Objetivo Geral

Identificar genes candidatos relacionados à resposta ao excesso de ferro em uma cultivar brasileira de arroz (BRS Querência) caracterizada como tolerante a este estresse.

3.3 Objetivos Específicos

- Obter sequências do transcriptoma de plantas de arroz via técnica de RNA-seq;
- Identificar os genes diferencialmente expressos entre o tratamento de excesso de ferro e tratamento-controle (condições normais de nutrição);
- Proceder à anotação dos genes diferencialmente expressos utilizando como referência os bancos de dados de sequência públicos de arroz;
- Fazer análise de enriquecimento e a classificação do *gene ontology* (GO) das sequências obtidas, além de detectar e identificar os metabolismos dos genes que tiveram sua expressão alterada;
- Identificar as possíveis interações proteínas-proteínas dos genes com expressão diferenciada nos bancos de dados;

- Analisar e identificar alterações nos padrões de *splicing* alternativo relacionados aos sítios de junções e nos diferentes tipos de eventos de *splicing*.

4 CAPÍTULOS

4.1 Artigo 1 – Dealing with iron metabolism in rice: from breeding for stress tolerance to biofortification

Artigo submetido à revista *Genetics and Molecular Biology*

Authors: Railson Schreinert dos Santos[1,2], Artur Teixeira de Araujo Júnior[1,2], Camila Pegoraro[1], Antonio Costa de Oliveira[1,2*]

[1] Plant Genomics and Breeding Center (CGF), Universidade Federal de Pelotas; Pelotas, RS, Brazil.

[2] Technology Development Center (CDTec), Universidade Federal de Pelotas; Pelotas, RS, Brazil.

*To whom correspondence should be addressed. Antonio Costa de Oliveira; Plant Genomics and Breeding Center, Universidade Federal de Pelotas; PO Box 354, Campus Universitário, S/N - CEP 96160-000, Capão do Leão, RS, Brazil.
E-mail: acostol@cgfufpel.org

Running title: Iron metabolism in rice

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Abstract

Iron is a well-known metal. Used since ancient times in many different ways this element is not less present in biology, where, unfortunately, it represents a two-way problem. Being an essential block in the composition of different proteins and metabolic pathways, iron is a vital component for animals and plants. That is why iron malnutrition represents a big problem with severe impact on the lives of different organisms, including humans, becoming a major concern, especially in developing countries. Ironically this metal is also capable of causing damage due to its excessive presence in certain places, being toxic to cells and affecting the whole organism. Because of its importance, much has been studied about the methods of iron absorption, transport and storage in order to design alternatives that may solve this problem. Here we will discuss how different strategies are used to circumvent the problem of lack and excess of iron in plants and possible impacts on human health and economy.

Introduction

Iron is the fourth most abundant element in the earth's crust, where ferric iron [Fe(III)] and ferrous iron [Fe(II)] are the most common forms (Hori *et al.,* 2015). While Fe(III) is insoluble and difficult to be absorbed by the roots, Fe(II) is soluble and readily available to plants. When the soil is aerated and in alkaline pH, Fe is oxidized into insoluble iron oxides, but in flooded soils, which are in anaerobic conditions, the pH decrease and there is a reduction of Fe(III) to Fe(II) (Morrissey and Guerinot 2009). This event is responsible for the low availability of Fe in upland soils and for its high availability in flooded soils.

Fe is an essential micronutrient for both animals and for plants. In mammals iron is part of the structure of a diversity of proteins (hemoglobin, myoglobin, cytochromes, flavoproteins, heme-flavoproteins, transferrin, lactoferrin, ferritin, hemosiderin, sulfur, non-heme enzymes) (Russel *et al.,* 2001). In plants Fe acts in photosynthesis, respiration and other important metabolic pathways (Briat and Lobreaux 1997; Kobayashi and Nishizawa 2012). Conversely, it also participates in the Fenton reaction, catalyzing the generation of hydroxyl radicals (OH), and reactive oxygen species (ROS) that can cause irreversible damage to the cell (Wu *et al.,* 2014). Thus, Fe stress can be caused by either deficiency and excess (Connolly and Guerinot 2002).

Iron deficiency can alter root morphology (Morrissey and Guerinot 2009), cause chlorosis of young leaves and reduce productivity (Kobayashi and Nishizawa 2014). To prevent the shortage of this element plants have developed two different absorption strategies: the Strategy I is used by higher plants, except for the family Poaceae. In this strategy the enzyme H^+ ATPase (AHA) mediates the release of hydrons and phenolic compounds from the roots to the rhizosphere, increasing the solubility of Fe(III). Also, in Strategy I, FERRIC REDUCTASE OXIDASE (FRO2) mediates the reduction of Fe(III) to Fe(II) and IRON REGULATED TRANSPORTER 1 (ITR1) is responsible for absorption of Fe(II) in the roots (Connolly and Guerinot 2002; Kobayashi and Nishizawa 2012).

The Strategy II, which is specific of grasses, is based on biosynthesis and secretion of compounds of the family of mugineic acids (MAs) called PHYTOSIDEROPHORES (PS), which are result of the action of NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT) and DEOXYMUGINEIC ACID SYNTHASE (DMAS). Due to the properties of the mugineic acid phytosiderophores and to the role of TOM1 as a transporter, MAs can bind to Fe(III) forming the soluble complex Fe(III)-MA. This complex is then transported into the cell by YELLOW STRIPE 1 (YS1) and YELLOW STRIPE1-LIKE (YSL) (Curie and Briat 2003; Nozoye *et al.*, 2011; Ricachenevsky *et al.*, 2011; Kobayashi and Nishizawa 2012). Rice (*Oryza sativa* L.) uses the Strategy II, but also presents the Strategy I, being able to absorb Fe (II) directly from the rhizosphere (Ishimaru *et al.*, 2006; Kobayashi and Nishizawa 2014).

The high level of Fe(II) found in some flooded soils can be toxic to plants (Mongon *et al.*, 2014). The toxicity caused by excessive Fe can occur directly and indirectly. The direct toxicity occurs when there is high uptake and excessive accumulation of this element in tissues followed by the appearance of brown-dark spots in the leaves (leaf-bronzing) (Becker and Asch 2005; Morrissey and Guerinot 2009). The indirect damage is caused by the prevention of the uptake, transport and utilization of other nutrients (eg.: P, K, Ca, Mg, Mn, and Zn) due to an accumulation of iron over the roots, a phenomenon known as ferric cover (Sahrawat 2004; Zhang *et al.*, 2014). Both situations affect plant growth, development and productivity, leading to significant yield losses. To adapt to this condition, rice plants have developed different mechanisms of tolerance (Type I, Type II and Type III) which are based on specific forms of use, exclusion and storage of iron (Figure 1). In Type I there is an oxidation and precipitation of Fe(II) on the root surface, while in Type II the absorption Fe (II), and storage occurs in a less reactive form in ferritin protein. Type III mechanism is based

in the tolerance to ROS formed in Fenton reaction (Wu *et al.*, 2014). An annulment of the uptaken Fe(II) through its storage in old or less active leaves or exclusion via symplast can also occurr (Becker and Asch 2005).

Physiological disorders caused by the excess of Fe are common in cultivated rice in the regions of Africa, Asia and South America (Shahid *et al.*, 2014). However, despite the high amount of Fe in the soil, which can even be toxic to the plant, little is accumulated in rice grains. In addition, the accumulation of iron in the grain occurs in the outermost layers being lost during the industrial processing (Sperotto *et al.*, 2012). Thus, rice contributes very little to meet the need of Fe intake in the human diet, not being an effective way of preventing anemia.

More than two billion people worldwide suffer from anemia, and more than 50% of these cases are caused by Fe deficiency (Arcanjo *et al.*, 2013). The iron-deficiency anemia (IDA) affects more dramatically the continents of Africa and Asia, where IDA is a major public health problem, prevalent in young women and children (Moretti *et al.*, 2006; Visser and Herselmamn 2013). Since it causes the death of almost one million people per year (Aung *et al.*, 2013).

Biofortification is an interesting strategy to solve the problem of IDA, especially for people who cannot change their eating habits due to financial, cultural or religious issues. In this sense we can not only increase the amount of iron in grains, but also decrease the content of inhibitors of Fe absorption commonly found in plants (Lucca *et al.*, 2001; Raboy 2002; Schuler and Bauer, 2011). In addition, biofortification is a sustainable strategy for the government. In this sense, rice can be the ideal species for biofortification, since it is a staple food that is especially important for developing countries, where IDA is even more severe. Also rice is grown in flooded soils, where Fe availability is higher (Becker and Asch 2005); and has its mechanisms of Fe uptake, translocation and homeostasis better understood than most of the species (Masuda *et al.*, 2012).

Global rice production is 741 million tons in approximately 165 million hectares. Rice is not only the second most cultivated cereal in the world, with important social and economic function, but is also an ideal model for functional genomics studies in monocots (FAOSTAT 2015; Yao *et al.*, 2015). The availability of different rice genomes of different subspecies has enabled the study of many genes and metabolic pathways.

Considering the importance of rice in nutrition and economy as well as the impact of iron deficiency and excess in the life of plants and animals in this review we will discuss the highlights of the uptake pathways, translocation, homeostasis and Fe accumulation in the grain to establish a link between Fe-toxicity and Fe-biofortification.

Identifying regulatory pathways

According to the availability of Fe in the soil, plants have developed mechanisms to control and regulate the absorption, translocation and subcellular storage of this mineral. Classical studies associated with the emergence of modern and advanced tools of genomics, transcriptomics and proteomics have enabled in-depth understanding of homeostasis of Fe in plants (Kobayashi and Nishizawa 2012). The uptake of Fe in plants occurs by using the Strategy I or reduction strategy (non Poaceae), the Strategy II or chelation strategy (Poaceae) and the combination of strategies I and II (rice) (Figure 2) (Ishimaru *et al.*, 2006; Zhang *et al.*, 2012; Yang *et al.*, 2013; Ricachenevsky and Sperotto 2014; Finatto *et al.*, 2015). The key genes involved in Strategy I are *AHA2* (protonation of the rhizosphere) *FRO2* [reduction of Fe(III) to Fe(II)] and *IRT1* (Fe(II) transport to inside the root) (Kim and Guerinot 2007; Hindt and Guerinot 2012).

In *Arabidopsis thaliana* (L.) Heynh there are eight homologues of *FRO* (*AtFRO1* to *AtFRO8*) while in *O. sativa* there are only two (*OsFRO1* and *OsFRO2*) (Victoria *et al.*, 2012). The gene *IRT* presents 15 homologues in *A. thaliana*, (*AtIRT1*, *AtIRT2*, *AtIRT3*, *AtZIP1* to *AtZIP12*) and 12 in *O. sativa* (*OsIRT1* to *OsIRT12*) (Ishimaru 2005; Kim and Guerinot 2007). *A. thaliana* presents 12 homologues of the gene *AHA* (*AtAHA1* to *AtAHA12*) (Santi and Schmidt 2009) and *O. sativa* ten (*OsA1* to *OsA10*) (Zhu *et al.*, 2009; Li *et al.*, 2015). Not all members of FRO, IRT, AHA families are directly involved with the capture of Fe.

In conditions of Fe deficiency there is an induction of the genes *AHA, FRO* e *IRT* (Hindt and Guerinot 2012). Studies conducted in *A. thaliana* demonstrate that the low availability of Fe leads to the induction of transcription factor (TF) FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR 1 (FIT1), which regulates *AtFRO2* at the level of mRNA accumulation and *AtIRT1* at the level of protein accumulation (Colangelo 2004). The co-expression of FIT with other TFs of the family *Basic helix-loop-helix* (*AtbHLH38/39*) directly regulate the expression of *IRT1* and *FRO2*, which causes an increase in iron accumulation (Yuan *et al.*, 2008; Hindt and Guerinot 2012). There are no orthologs of FIT in

rice, but *AtbHLH38/39* are similar to *OsIRO2* (Hindt and Guerinot 2012) that regulates genes related to transport of Fe(III)-MA, but does not regulate *OsIRT1* (Ogo *et al.,* 2007).

The *FIT* gene is regulated by signaling molecules as auxin and ethylene, synthesized in conditions of iron deficiency. In *Arabidopsis* the lack of Fe induces an increase in auxin synthesis, resulting in increased expression of the genes *FIT* and *FRO2* (Chen *et al.,* 2010). Similarly to what happens to auxin, an increase in ethylene synthesis is also noticed under these conditions, an event that causes the up regulation of *FIT* (Lucena *et al.,* 2006) and therefore of *FRO* and *IRT*. The product of *FIT* gene reacts with the TFs ETHYLENE INSENSITIVE 3 (*AtEIN3*) and ETHYLENE INSENSITIVE 3–LIKE1 (*AtEIL1*) emphasizing the importance of ethylene signaling in response to Fe deficiency (Lingam et al., 2011). Ethylene mediated response to iron excess may also play a role in the differential regulation of TFs of the ERFs family, which can also be modulated when rice plants are subjected to this stressful condition (Santos et al., 2013).

Just as auxin and ethylene, nitric oxide (NO) has its synthesis increased in conditions of Fe deficiency. NO acts as a positive regulator of genes whose products act on Fe uptake (Hindt and Guerinot 2012). Conversely, under conditions of Fe excess the three *IRT* genes and *OsFRO2* are induced in rice (Finatto *et al.*, 2015).

In *A. thaliana* the POPEYE (AtPYE) and BRUTUS (AtBTS) TFs also participate in the regulation of genes associated with Fe absorption. These TFs act in sensitivity and root response to the availability of Fe and the interaction of these TFs with other regulatory proteins involved in regulating the homeostasis of Fe (Long *et al.,* 2010). In rice the genes *OsIRO3* (Zheng *et al.,* 2010) and *OsHRZ1/OsHRZ2* (Kobayashi *et al.,* 2013), have been identified. *IRO3* is an ortholog of *AtPYE,* and *HZR1* e *HZR2* are orthologs of *AtBTS.*

The Strategy II (Figure 2) includes the participation of genes that act in the cycle of MAs precursors - METHIONINE and S-ADENOSYL-L-METHIONINE (*5′-METHYLTHIOADENOSINE NUCLEOSIDASE – MTN*, *METHYLTHIORIBOSE KINASE – MTK*, *METHYLTHIORIBOSE-1-PHOSPHATE ISOMERASE – IDI2* and *DEHYDRASE ENOLASE PHOSPHATASE* – DEP, *S-ADENOSYL-L-METHIONINE SYNTHETASE – SAMS*) (Kobayashi *et al.,* 2005; Suzuki *et al.,* 2006), in the synthesis of MAs (*NAS*, *NAAT*, *DMAS*, *Dioxygenases – IDS2/IDS3*) (Nakanishi *et al.,* 2000; Kobayashi and Nishizawa 2012), binding of MAs to Fe(III) (*TRANSPORTER OF MUGINEIC ACID PHYTOSIDEROPHORES 1 – TOM*1) (Nozoye *et al.,* 2011), and in the transport of the complex Fe(III)-MAs into the root

(*YS1* e *YSL*) (Curie *et al.*, 2001; Inoue *et al.*, 2009; Kobayashi and Nishizawa 2012). Four homologues of the gene *NAS* are present in the genome of *A. thaliana* (*AtNAS1*, *AtNAS2*, *AtNAS3* and *AtNAS4*) and three in the rice genome (*OsNAS1*, *OsNAS2* and *OsNAS3*) (Victoria *et al.*, 2012). Six homologues of the *NAAT* gene (*OsNAAT1* to *OsNAAT6*), and only one *DMAS* gene (*OsDMAS1*) are present in rice (Bashir *et al.*, 2006; Broadley *et al.*, 2010). For *YSL*, eight homologues were identified in *A. thaliana* (*AtYSL1* to *AtYSL8*) and 18 in rice (*OsYSL1* to *YSL18*) (Victoria *et al.*, 2012).

As well as the genes involved in Strategy I, genes associated with Strategy II are induced in iron deficiency (Ricachenevsky and Sperotto 2014). The TFs IRON DEFICIENCY RESPONSIVE ELEMENT BINDING FACTOR 1 (IDEF1 e IDEF2*)* and IRON REGULATED BASIC HELIX-LOOP-HELIX (IRO2) have been identified as regulators of key genes which control Fe uptake, including the synthesis of MAs in rice (Itai *et al.*, 2013). Fe deficiency the OsIDEF1 up regulates genes whose products act in capture and use of Fe in rice, as *OsIRO2*, *OsYSL15*, *OsYSL2*, *OsIRT1*, *OsNAS1*, *OsNAS2* and *OsNAS3* (Kobayashi *et al.*, 2009). The TF IDEF1 binds to iron deficiency-responsive elements (IDE1 and IDE2), which are present in the promoter region of genes associated with Fe deficiency (Kobayashi *et al.*, 2007). Moreover, *OsIRO3* is induced in Fe deficiency and acts as a negative regulator of genes related to this condition in rice (*OsNAS1*, *OsNAS2*, *OsIRO2*, *OsIRT1*, *OsYSL15* and *OsNRAMP1*) (Zheng *et al.*, 2010).

In conditions of Fe toxicity the genes *OsNAS1*, *OsNAS2*, *OsYSL15*, *OsYSL16* and *OsNARAMP1* were repressed in rice roots (Quinet *et al.*, 2012). In a similar study, Finatto *et al.*, (2015) reported the induction of the genes *OsNAAT1*, *OsYSL1* and *OsYSL17* in rice plants grown under excessive Fe.

After Fe capture by the roots, the element is transported to other organs, a process that involves several steps, passing through symplast, xylem (transpiration stream) and phloem (Kim and Guerinot 2007). When Fe enters the symplast it is oxidized and ligated to chelating molecules. Chelators that Fe can bind to are citrate, NICOTIANAMINE (NA) (Figure 3) and MAs (Kobayashi and Nishizawa 2012), being citrate the most important one (Curie and Briat 2003). In *A. thaliana* the gene *FERRIC REDUCTASE DEFECTIVE 3* (*AtFDR3*) encodes a transmembrane protein belonging to the family of Multidrug and toxin efflux transporters (MATE) that facilitates the transport of citrate in the xylem (Durrett *et al.*, 2007).

In rice, *OsFRDL1* is required for efficient translocation of Fe-citrate complex (Yokosho *et al.,* 2009). In rice plants under conditions of Fe excess, the induction of three genes belonging to MATE family, which may be involved in reducing ROS production in mitochondria, was observed (Finatto *et al.,* 2015). Genes belonging to *YSL* and *IRT* families, as well are not only involved in iron uptake, but also in the transport of this element trough the plant. Different *YSL* genes carry different complexes. In rice for example, *OsYSL2* carries Fe(II)NA (Koike *et al.,* 2004) while *OsYSL15* product carries Fe(III)-DMA (Lee *et al.,* 2009). *OsIRT1* was present not only in roots, but also in rice leaves and stems, indicating its participation in the Fe transport over long distances (Narayanan *et al.*, 2007).

To be assimilated by the leaves, Fe(III) is reduced trough the action of FRO enzymes. *LeFRO1* in *Lycopersicum esculentum* Mill. (Waters *et al.,* 2002), *PsFRO1* in *Pisum sativum* L. (Li *et al.,* 2004) and *AtFRO6* in *A. thaliana* are expressed in the aerial part of the plant (Feng *et al.,* 2006) indicating their participation in the reduction of Fe(III). After reduction, Fe is transported to other organs of the plant. This transport is made via the phloem NICOTIANAMINE carrier (Takahashi *et al.,* 2003), which is synthesized through the enzyme NICOTIANAMINE SYNTHASE (NAS). The Fe transport also occurs trough family members of the Natural Resistance-Associated Macrophage Protein (NRAMP) (Nevo and Nelson 2006). NRAMP carriers are related to subcellular transport of Fe and its segmentation in vacuoles and/or plastids (Curie *et al.,* 2000). Six counterparts NRAMP were detected in *A. thaliana* and eight in rice (*OsNRAMP1-OsNRAMP8*) (Victoria *et al.,* 2012).

In rice, *OsNRAMP1* is expressed mainly in roots, *OsNRAMP2* in leaves and *OsNRAMP3* is expressed in both tissues (Belouchi *et al.,* 1997). In conditions of Fe excess, Quinet *et al.,* (2012) noticed the repression of the gene *OsNRAMP1* while Finatto *et al.,* (2015) observed the induction of another NRAMP gene, *OsNRAMP6*.

Inside the cell, Fe can be incorporated into proteins or stored in plastids and mitochondria, where it is found associated with ferritin (Duy *et al.,* 2011; Vigani *et al.,* 2013), or even in the cell vacuole (Gollhofer *et al.,* 2014) (Figure 2). This compartmentalization is performed aiming the homeostasis of Fe, especially in conditions of excess of this element. Ferritin is an iron storage protein, whose main function is to provide Fe for protein synthesis and avoid damage caused by free radicals produced by the interaction iron/dioxygen (Goto *et al.,* 1999). This protein has the ability to store more than 4500 Fe atoms in a soluble, non-toxic and bioavailable form (Briat and Lobreaux, 1997). In *Oryza glaberrima* Steud. and *O. sativa* the

tolerance to Fe toxicity seems to be associated with ferritin synthesis (Majerus *et al.,* 2007; Silveira *et al.,* 2009). However, ferritin stores only a small portion of Fe, being the vacuole the main compartment for accumulation of this element (Gollhofer *et al.,* 2014). *A. thaliana* has four homologues of ferritin encoding genes (*AtFER1* to *AtFER4*) while in *O. sativa* two of these can be found (*OsFER1* and *OsFER2*) (Silveira *et al.,* 2009). The Fe-dependent regulation of *AtFER1* and *ZmFER1* gene depends on the presence of a *cis*-element called Iron-dependent Regulatory Sequence (IDRS) in their promoter regions. The IDRS element is involved in the repression of FER genes in plants that are under low concentrations of Fe (Petit *et al.,* 2001). In Fe excess the genes *OsFER1* and *OsFER2* show increased amount of these transcripts, being *OsFER2* preferably up regulated (Stein *et al.,* 2009). Similar results were found by Quinet *et al.,* (2012), which also observed the induction of *OsFER1* and *OsFER2* genes in stress by excess of Fe in the soil. In other species it has also been observed the induction of genes FER in toxic amounts of Fe.

Vacuoles are multifunctional organelles dynamically adjusted according to environmental conditions. This organelle has buffering capacity serving as a reservoir of metabolites, minerals, nutrients and also as a deposit for toxic compounds, being crucial for the process of detoxification and for cellular homeostasis (Marty, 1999; Peng and Gong, 2014). The uptake of Fe by the vacuole is mediated by FERROPORTIN (FPN) (Morrissey and Guerinot, 2009) and by members of a family of Vacuolar Iron Transporters (VIT) (Zhang *et al.,* 2012). In *A. thaliana* three homologues of FPN (*AtFPN1*/*AtIREG1*, *AtFPN2/AtIREG2* and *AtFPN3/AtIREG3*) were found (Curie and Briat, 2003; Morrissey and Guerinot, 2009; Merlot *et al.,* 2014), while in *O. sativa* only two of these genes (*OsFPN1/OsFerroportin; OsFPN2/IREG3*) were detected (Bashir *et al.,* 2011; Merlot *et al.,* 2014). In *A. thaliana* the iron accumulation in the vacuole of seed cells depends on *AtVIT1* (Kim *et al.,* 2006). In rice the membrane transporters encoded by *OsVIT1* and *OsVIT2* genes are involved in the translocation of Fe between flag leaf and seeds, and inhibition of those result in increase of Fe in the seed, suggesting that new mechanisms are activated under this condition (Zhang *et al.,* 2012), and under conditions of excess Fe, *OsVIT1* had to be increased (Finatto *et al.,* 2015). Fe remobilization from the vacuole to the cytoplasm is mediated by NRAMP3 and NRAMP4 (Peng and Gong, 2014).

Quantitative Trait Loci

In anaerobic conditions high amounts of Fe(II) are absorbed by plants, resulting in the accumulation of this element in the cell (Santos and de Oliveira 1994). In rice, there is a differential response between cultivars to stress by Fe excess. When both susceptible and tolerant cultivars, BR-IRGA 409 and EPAGRI 108 respectively, are subjected to high concentrations of Fe there was less accumulation of this element and greater accumulation of the protein ferritin in the tolerant cultivar, suggesting that ferritin protein may be involved in this mechanism of tolerance (Silveira *et al.,* 2009). However, it was found that when there is Fe accumulation, the activity of aconitase enzyme and protein ferritin levels are higher in a genotype that accumulates higher concentrations of Fe when compared to a lower accumulator genotype (Panda *et al.,* 2014). Conversely, a previous study showed that the accumulation of iron is not parallel to the level of ferritin expression in rice seeds overexpressing the *SoyFER* gene (of soybean ferritin), suggesting that the Fe accumulation may be limited by uptake and transport of this element (Qu *et al.,* 2005). According to these studies it was found that the mechanisms associated with tolerance to toxicity and accumulation of Fe is not well understood. However, studies related to the identification of Quantitative Trait Loci (QTLs) and genes whose products are responsible for the homeostasis of Fe and the accumulation of this mineral in the grain have been conducted (Figure 4) and the results of these surveys can assist breeding programs for toxicity and biofortification for Fe content (Wu *et al.*, 1998; Wan *et al.,* 2003; Dufey *et al.*, 2009; Shimizu A. 2009; Wu *et al.,* 2014). For this feature, three loci were located on chromosomes 7, 8 and 9 rice explaining around 19-30% of the difference in the concentration of Fe in the grains (Gregorio *et al.,* 2000). The higher concentrations of Fe in the grain were positively correlated with the expression of genes *OsYSL14*, *OsNAC5* and negatively correlated with *OsNARAMP7*, *OsNRAMP8* and *OsFRO1* expression (Sperotto *et al.*, 2010). On the other hand, *OsFER1*, *OsNRAMP4*, *OsNRAMP5*, *OsNRAMP6*, *OsYSL6*, *OsYSL12*, *OsYSL4*, *OsZIP8*, *OsZIP10* expression was correlated with higher concentrations of Fe in the grain (Banerjee and Chandel, 2011). The functional characterization of these genes can help in getting biofortified rice genotypes with higher concentrations of Fe in the grains. In QTL analysis for tolerance to bronzing, using a F3 population from the cross between cv. Gimbozu (*japonica* genotype which is tolerant to Fe excess) and cv. Kasalath (*indica* genotype which is susceptible to Fe excess) seven QTLs associated with this feature were detected. These QTLs which are located on chromosomes 1, 2, 7, 8 and 12, explain 99% of the phenotypic variation for bronzing and

showed no detectable epistatic effect (Shimizu, 2009). In a population generated from the cross between cv. Azucena (tolerant *japonica*) and cv. IR64 (susceptible *indica*), a QTL on chromosome 1 that is associated with leaf bronzing index was detected (Dufey *et al.,* 2009). The association of this region with bronzing index had already been detected earlier by Wan *et al.,* (2003) and Wu *et al.,* (1998). Also in a QTL analysis in a population obtained from the cross between cv. Kasalath (susceptible *indica*) and cv. Koshihikari (tolerant *japonica*) researchers found a QTL on chromosome 3 associated with Fe concentration in the shoot (Fukuda *et al.,* 2012). In a study conducted by Wu *et al.* (2014), populations from the crosses IR29 (susceptible *indica*) x Pokkali (tolerant *indica*) and Nipponbare (moderately tolerant *japonica*) x Kasalath (highly susceptible *japonica*) were used for identification of QTLs associated with tolerance to Fe excess. In the population IR29/Pokkali, seven QTLs for leaf bronzing were identified, located on chromosomes 1, 2, 4, 7 and 12, explaining 9.2 to 18.7% of the phenotypic variation. In a Nipponbare/Kasalath/Nipponbare backcross inbreed population three QTLs were mapped on chromosomes 1, 3 and 8, and these QTLs explain 11.6 to 18.6% of the phenotypic variation. Additional studies demonstrated that the QTL on chromosome 1 was associated with shoot tolerance and the QTL on chromosome 3 was associated with exclusion of Fe in roots. Similarly to the QTL studies for stress tolerance to Fe much effort has been made in identifying QTLs associated with Fe content in grains. Four QTLs for Fe accumulation (*qFe1*, *qFe3*, *qFe4* and *qFe7*) located on chromosomes 1, 3, 4 and 7, accounting, respectively, for 16.2%, 21.4%, 9.7% and 15.5% of the phenotypic variation, were found in a F6 population from the cross cv. Bala (*indica*) x Azucena (*japonica*) (Norton *et al.,* 2010). Using Composite Interval Mapping on an F6 population from the cross Madhukar × Swarna it was possible to identify 7 QTLs associated with iron accumulation (*qFe1.1*, *qFe1.2*, *qFe5.1*, *qFe7.1*, *qFe7.2*, *qFe12.1* and *qFe12.2*), which are located on chromosomes 1, 5, 7 and 12 (Anuradha *et al.,* 2012). The candidate genes for QTLs are: *OsYSL1* (LOC_Os01g13710) which are located within the *qFe1.2*; *OsMTP1* (LOC_Os05g03780) located within the *qFe5.1*; *OsNAS3* (LOC_Os07g48980) located within the *qFe7.1* and *qFe7.2*; *OsNRAMP1* (LOC_Os07g15460) located within the *qFe7.2*; and *OsZIP8* (LOC_Os07g12890) located 0.3 Mb right of *qFe12.1*. Most phenotypic variance was explained by the QTL on chromosome 12 (71%) (Anuradha *et al.,* 2012).

Mapping of a population derived from the cross Chunjiang 06 (*japonica*) x TN1 (*indica*) detected 3 QTLs for Fe accumulation in grains. The QTLs are located on chromosomes 1, 6 and 8, explaining, respectively 15.7, 10.6 and 22.3% of the phenotypic variation for Fe

accumulation in the grains (Du *et al.*, 2013). A QTL for Fe concentration on chromosome 8, was detected in a population from the cross cv. Lemont (*japonica*) x cv. TeQing (*indica*) (Zhang *et al.*, 2014). A collection of Dale Bumpers National Rice Research Center, USDA ARS, Stuttgart, AR, USA composed by 221 accesses of *O. sativa*, 5 accesses of *O. glaberrima*, 2 accesses of *O. rufipogon* and 1 of *O. nivara* have been mapped aiming the identification of QTLs for contents of different minerals in the grain (Nawaz *et al.*, 2015). In this study, the authors identified 11 genetic regions responsible for binding and transport of Fe, comprising the genes *OsZIP1* (Os01g0972200), *OsHMA4* (Os02g0196600), *OsACA2* (Os02g0176700), *OsZIP2* (Os03g0411800), *OsCNGC* (Os03g0758300), *OsZIP3* (Os04g0613000), *OsZIP5* (Os05g0472700), *OsZIP9* (Os05g0472400), *OsHMA2* (Os06g0700700), *ABC TRANSPORTER* (Os06g0607700), *OsNAS3* (Os07g0677300), *HEAVY METAL TRANSPORTER* (Os07g0671400), *CHY ZINC FINGER* (Os10g0456800) and *OsACA9* (Os12g0136900). In *A. thaliana*, two QTLs were identified in a region on chromosomes 1 and 5 in which genes (*ZIP10* and *NAS1*) are associated with the Fe metabolism, playing a role in cation translocation (Vreugdenhil *et al.*, 2004). Although further studies are required for the elucidation of mechanisms and genes related with the increase of iron concentration in seeds and stress tolerance to Fe excess, much work has already been developed in QTL mapping and its association with other metabolic pathways (Wan *et al.*, 2003; Shimizu *et al.*, 2009).

Phylogeny

A phylogenetic study on members of gene families related to homeostasis of Fe (NAS NRAMP, YSL, FRO and IRT) was conducted in *O. sativa*, *A. thaliana*, *Physcomitrella patens* (Hedw.) Bruch & Schimp. and other monocots and dicots (Victoria *et al.*, 2012). In this study, the authors found that the FRO genes can be grouped into two clusters, but these do not separate monocots, dicots and bryophytes, a first clue indicating that the divergence of these genes occurred even before the diversification of land plants. Conversely, for *NAS* genes it was observed the formation of a group with monocots and dicots. In the IRT family the genes were grouped into different clusters that separates monocots, dicots and bryophytes. For NRAMP genes, no evidence for divergence between groups of plants was observed, since genes from monocots and dicots were together in different clusters. Finally, the authors found

658 that *YSL* genes possibly went through two duplication events, which probably occurred before
659 the divergence of monocots and dicots.

660 Phylogenetic analyzes were also performed by Gross *et al.,* (2003). In this study they
661 analyzed a total of 43 genes belonging to five families: YS, FRO, ZIP, NRAMP, and ferritin
662 proteins. The analysis of the YS family shows a relationship between predicted members of
663 rice, *Arabidopsis* and *Zea mays* L., indicating that the putative new genes were homologous to
664 maize YS, indicating that these may also have a role in Fe transport. The proteins from family
665 FRO were separated from the burst oxidases, having a subdivision of FRO sequences, having
666 *OsFRO1* in a group and the *OsFRO2* in another group. Members of the ZIP family were
667 grouped in a single tree, with the *OsZIP1* and *OsZIP6* more distantly related. The NRAMP
668 family was divided into two classes, one most similar with *AtNRAMP1* and another with
669 *AtNRAMP2*, in which the number of exons is determinative in grouping these sequences. The
670 ferritin family had a separation between each of the species analyzed, where mammalian
671 ferritins were separated from their respective homologues. The separation was also noticed
672 between monocots and dicots, first we can observe the divergence of *Arabidopsis* genes and
673 just then genes from maize and rice diverge one from each other.

674

675 **Strategies for Fe biofortification in rice**

676 Biofortification is a process that increases the bioavailability of essential elements in the
677 edible part of plants (White and Broadley, 2005; Zielińska-Dawidziak, 2015). Although Fe is
678 the fourth most abundant element in the earth's crust, little of this element is available for
679 human nutrition through grains (Kim and Guerinot, 2007), something that contributes to rank
680 iron deficiency as the sixth risk factor for death and disability (WHO, 2015).

681 Although rice is a widely consumed food, it is not a rich source of iron, furthermore most of
682 the Fe content of rice grains is accumulated in the aleurone and in the embryo, two parts that
683 are lost during milling. After that, grains consist almost in its entirety by the endosperm,
684 losing up to 80% of the iron content and constituting a poor source of Fe for the human diet.
685 This makes the evaluation of iron content in polished and unpolished grains an important
686 information when studying biofortification (Brinch-Pedersen *et al.,* 2007; Paul *et al.,* 2012;
687 Bashir *et al.,* 2013b).

Among the plant breeding methods, transgenesis has high potential for Fe biofortification since this is a fast and efficient technique which is already being used for this purpose. Studies on rice biofortification by Fe using transgenics were conducted using five different strategies. In the first strategy, the increase in the amount of Fe in the grain was achieved through the expression of the soybean *FERRITIN* gene coding (*SoyFERH1*) under the control of *GLUTELIN* gene promoter from rice (*OsGluB1*), which is specific for the endosperm. The higher expression of FERRITIN in the endosperm resulted in at least 2-fold increase of Fe in *japonica* cv. Kitaake (Goto *et al.*, 1999), and in *japonica* cv. Taipei 309 (Lucca *et al.*, 2001). The increase was of 3.7-fold in the *indica* cv. IR68144 (Vasconcelos *et al.*, 2003) and 2.1-fold in the *indica* cv. Pusa Sugandhi II (Paul *et al.*, 2012).

In the second strategy, the increase in the amount of Fe in the grain was due to the overexpression of genes involved in the synthesis of mugineic acid. When overexpressing *NICOTIANAMINE SYNTHASE* (*NAS*) it was possible to notice an increase of even more than 3-fold in Fe content of polished grains of the *japonica* cultivars Tsukinohikari (Masuda *et al.*, 2009), Dongjin (Lee *et al.*, 2009), and Nipponbare (Johnson *et al.*, 2011). When *DIOXIGENASE* (*IDS3*) was overexpressed it caused an increase of 1.4-fold in Fe content of polished grains of the *japonica* rice Tsukinohikari (Masuda *et al.*, 2008).

In the third strategy the gene *OsYSL2* was inserted under the control of the promoter of *SUCROSE TRANSPORTER* (*OsSUT1*), resulting in increased expression of this gene in panicle and grains. This transformation increased in 4.4-fold the concentration of Fe in polished grains of the *japonica* cultivar Tsukinohikari (Ishimaru *et al.*, 2010). In the fourth strategy there is a combination of the first three strategies, generating the rice "*Fer-NAS-YSL2*", which presented a 4 to 6-fold increase in Fe content of polished grains in the *japonica* cv. Tsukinohikari (Masuda *et al.*, 2012) and 3.4-fold increase in the other *japonica* cv. Paw Yin San (Aung *et al.*, 2013).

In the fifth strategy, besides increasing the Fe content in the grain, it was sought to increase tolerance to Fe deficiency as well. In this case, a concurrent insertion of *SoyFERH2* gene was used, under the control of promoters of *OsGluB1* and *OsGlb*, and also the *HvNAS1* genes *NICOTIANAMINE AMINOTRANSFERASE* (*HvNAAT-A* and *HvNAAT-B*) and *MUGINEIC ACID SYNTHASE* (*IDS3*) of barley, which encode enzymes of the biosynthesis of MAs. Here the transformed plants showed to be tolerant to Fe deficiency and also to be capable of accumulating 2.5 to 4-fold of this mineral in polished grains (Masuda *et al.*, 2013).

Also the overexpression of the gene *OsIRT1* using a constitutive promoter (maize ubiquitin), resulted in higher concentration of iron and zinc in shoots and roots and an increase in tolerance to iron deficiency at the seedling stage. It was also possible to detect an increase in the concentration of these metals in mature grains with 13% more iron and 12% more zinc (Lee and An, 2009).

Similar data was found in plants overexpressing *OsIRO2*. These plants showed to be more tolerant to iron deficiency and presented an increase in Fe content in shoots (2-fold increase) and grains (more than twice) to grown in calcareous soil (Ogo *et al.*, 2011).

In addition, another strategy used is through the knockdown of the gene *OsVIT2*. Bashir *et al.* (2013b) showed that transgenic plants *OsVIT2-knockdown* had an increase in the concentration of iron in polished grains 1.8-fold. This suggests that the disruption of this gene helps in increasing the amount of iron in the grains, constituting a possible strategy for producing biofortified rice.

Although the strategies using transgenics resulted in an increase in grain Fe content, it is known that the polishing process is still responsible for major losses of this mineral. However, we should not forget that, according to the genotype, the location of Fe in the grain may vary (Sperotto *et al.*, 2010). Thus, further studies should be conducted aiming to develop new strategies for internalization of Fe in the grain, something that can be combined with other strategies.

The flag leaves are the main source of photoassimilates for the development of seeds in rice. The Fe concentration of the flag leaf decreased during the reproductive development in rice whereas the iron content of the grains increased. An interesting fact is that the cultivar with lower Fe accumulation in the grains showed higher Fe accumulation in flag leaf. This study demonstrates that there is an iron remobilization from the flag leaf to the grains, and increasing this remobilization can help us in obtaining biofortified grains (Sperotto *et al.*, 2010).

Another biofortification alternative to decrease the content of inhibitors of Fe absorption, such as phytic acid (Raboy, 2002), or to increase the activity of phytase (Lucca *et al.*, 2001). The antisense repression of *1D-MYO-INOSITOL-3-PHOSPHATE SYNTHASE* gene, encoding a key enzyme in the synthesis of phytic acid, resulted in 68% reduction of phytic acid content (Kuwano *et al.*, 2009).

The commercialization of genetically modified Fe biofortified crops has some limitations, either by farmers (changes in the appearance of the product) and consumers (high cost and acceptance of genetically modified organisms). In this sense, methods based on the selection of genotypes that are rich in Fe, followed by hybridization, can be better accepted (Zielińska-Dawidziak, 2015).

Rice Germplasm banks can be screened to identify genotypes that can absorb and store Fe more efficiently, so more QTLs related to these characteristics can be mapped and introgressed into elite varieties. In this case, it is taken into account the natural variation that occurred during evolution, taking advantage of the effects of specific interactions between different genes and alleles (Schuler and Bauer, 2011). As an example of the potential for exploitation of these banks we can cite the four-fold difference found when comparing the iron content of aromatic and traditional varieties (Mulualem, 2015).

The natural variation related to Fe accumulation in rice grains that has been already detected is quite low, in addition, grinding and polishing the grains results in a loss of up to 80% of this element, since Fe is stored primarily in the embryo and aleurone layer and not in the endosperm (Brinch-Pedersen *et al.,* 2007). Furthermore, the Fe concentration is deeply influenced by the interaction between genotype and environment (Graham *et al.,* 1999). However, despite these limitations, the International Rice Research Institute (IRRI) has developed the cultivar IR68144, which has about twice the concentration of Fe of other local varieties (Gregorio *et al.,* 2000).

The development of cultivars with increased iron content in the grains, even at relatively low levels, associated with results of the characterization of 1138 genotypes, that identified a variation of 6.3 to 24.4 $\mu g.g^{-1}$ of Fe in grains, suggests that there is genetic potential for development of other new varieties with high amounts of Fe (Gregorio *et al.,* 2000; Mulualem, 2015). Furthermore, the genetic variability for the content of phytic acid can also be exploited. These possibilities make the future of genetic progress seem really optimistic (Liu, 2005).

Conclusions

Being essential in the composition of different proteins and metabolic pathways, iron is vital for animal and plant health. Actually it is really ironic that an element capable of generating

toxic effects due to its high bioavailability in certain places is also a problem due to its low availability in others. To solve this problem, studies aiming the identification and understanding of pathways related to the regulation of iron metabolism are being conducted, combined with molecular markers in the identification of QTLs that may act on these pathways, and the use of phylogeny to better understand the evolution of these, aiming to decrease the sensitivity of rice to the lack and to the excess of iron in the soil, as well as to help in the generation of biofortified plants with higher iron content in the grains.

Throughout these studies we can see that we have succeeded not only in the description of the regulatory pathways, but also in breeding for improved varieties. Advances continue to be made and obstacles being overcome. In the future we should, besides pyramiding QTLs related to iron tolerance and increased iron content in grains, also focus on the exploration of the existing variation for genes that have proven to be important in experiments involving transgenic analysis, this should enable us to achieve greater market acceptance and to reduce bureaucratic obstacles, which greatly hinder the release of genetically modified organisms.

Although the genetic progress may seem difficult at certain times, our ability to deal with iron metabolism in rice has increased, and soon we should obtain cultivars that will be highly tolerant to iron stress, both by excess and a lack of this mineral. Allied to this, we should also be able to develop biofortified plants with higher content of iron in their grains, helping the fight against anemia and providing better quality of life especially for those who are not favored by our political system.

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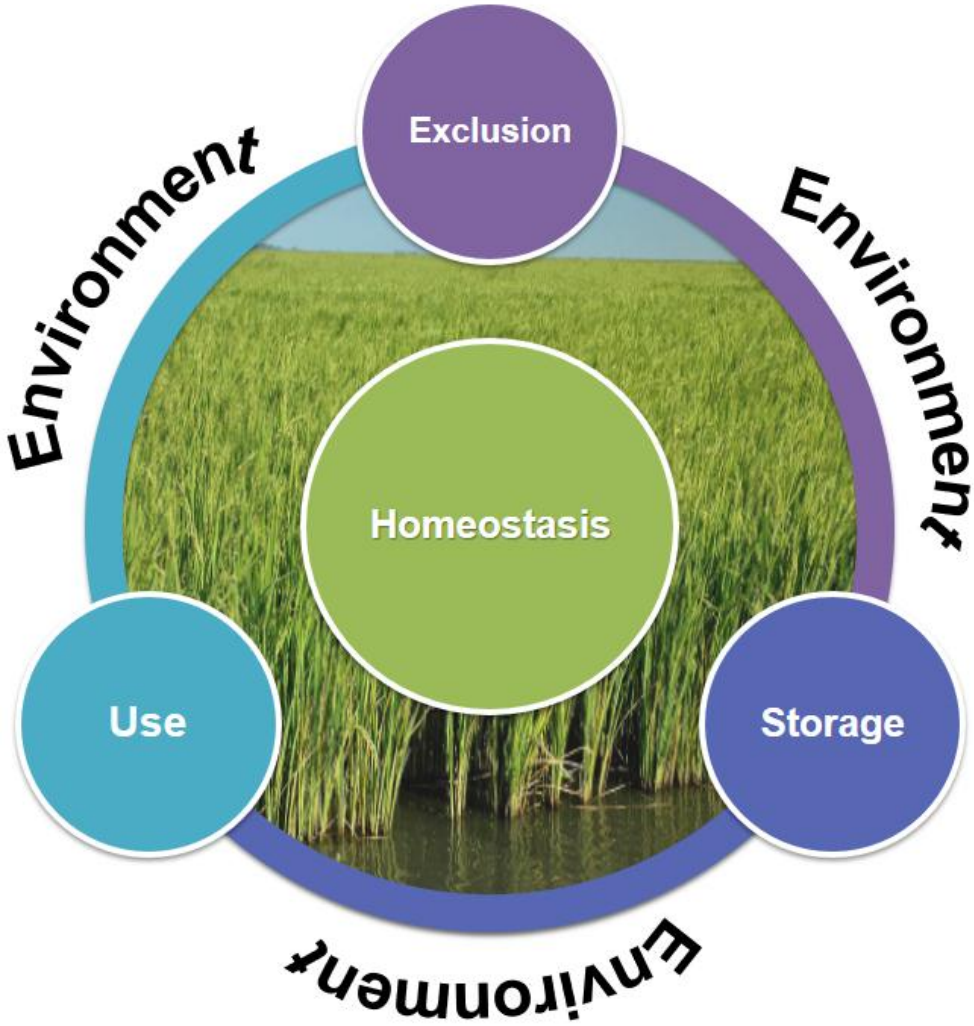

Figure 1. Mechanisms of iron homeostasis in plants. Survival under conditions of iron excess depends on the ability to use, exclude and/or store this element. These are deeply dependent on both genetic and environmental factors. Adapted from Perdiguero and Muñoz-Cánoves (2008).

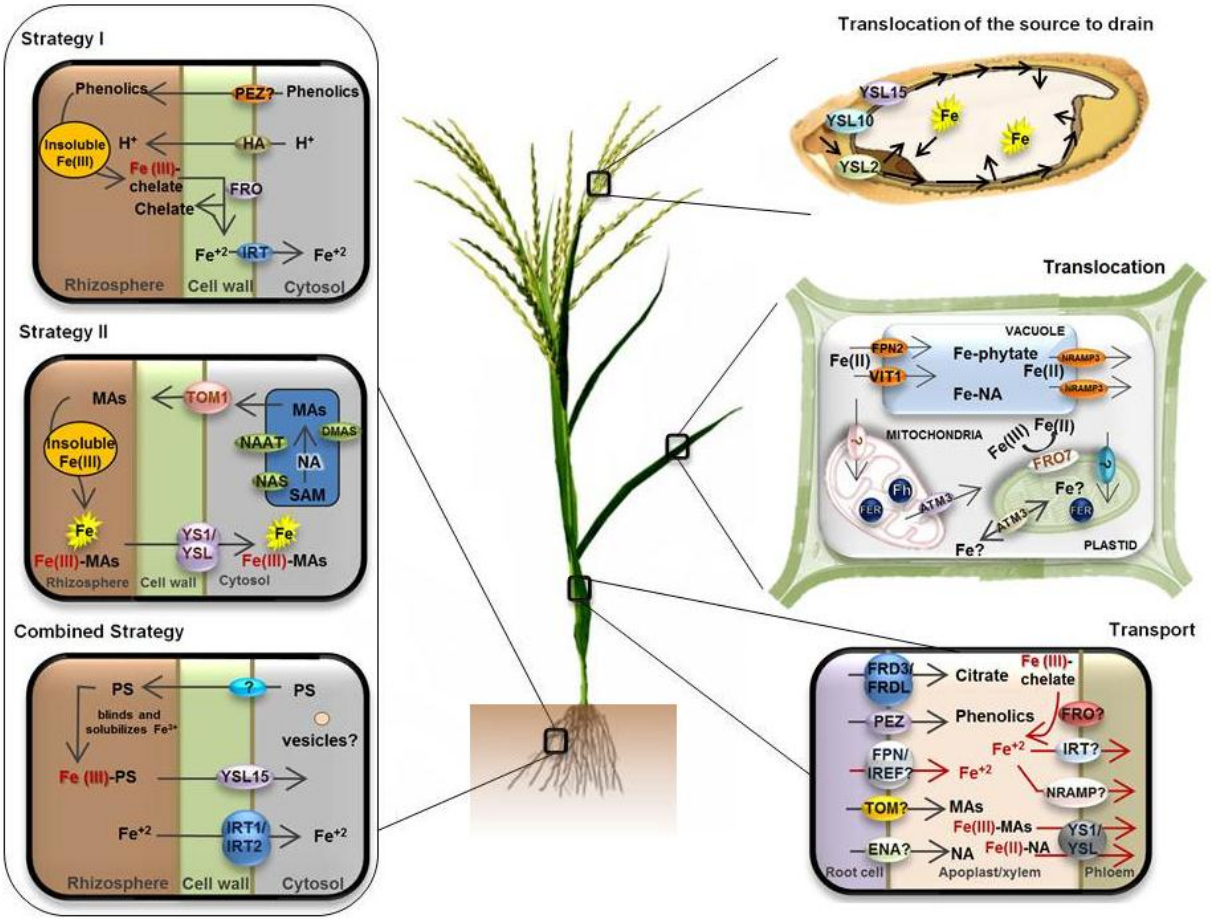

Figure 2. Absorption and translocation of iron in rice. Adapted from Palmer and Guerinot (2009); Kobayashi and Nishizawa (2012); Bashir *et al*. (2013a).

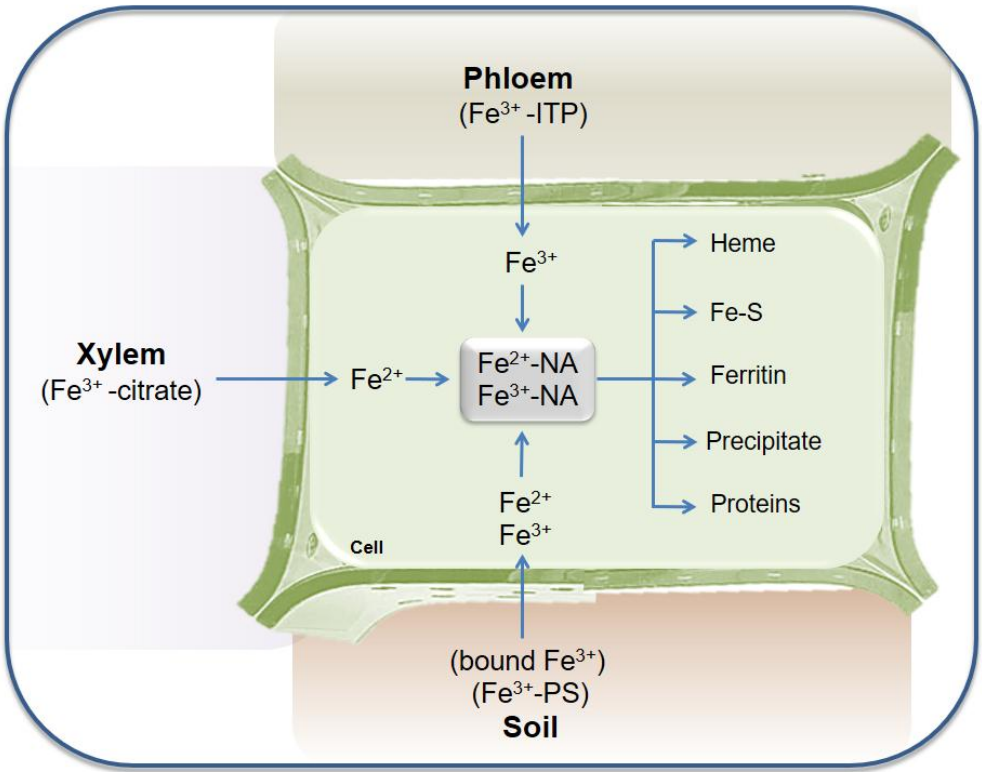

Figure 3. Role of nicotianamine (NA) in iron metabolism in plant cells. Iron can enter the plant cell through various strategies depending on the nature of the iron source. In this context NA is an important chelator that is able to provide iron in a functional form, avoiding precipitation and catalysis. Adapted from Hell and Stephan (2003).

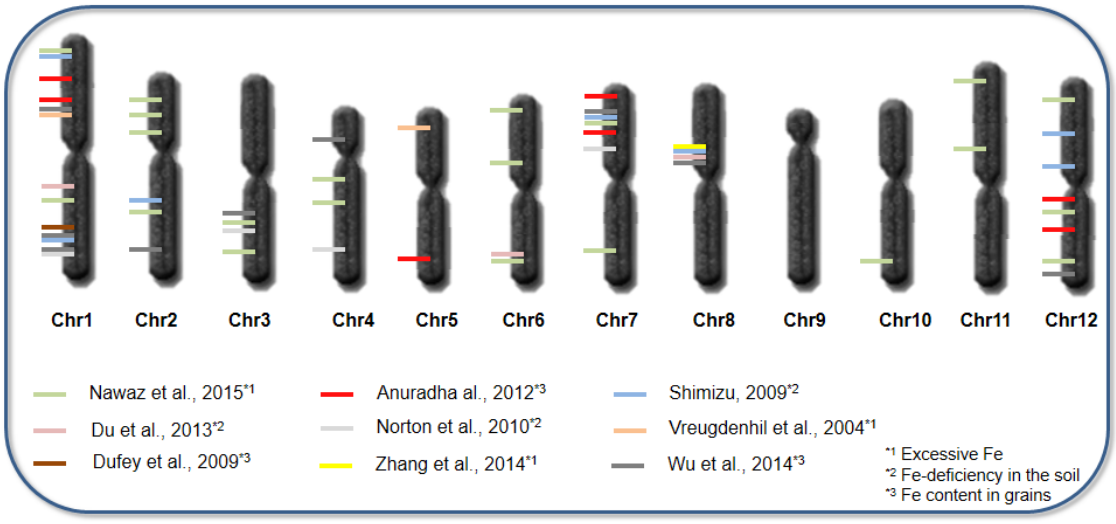

Figure 4. QTLs related to Fe metabolism. Map with the location of different QTLs related to tolerance to low and/or excessive amounts of Fe in the soil, and/or related to the variation of Fe content in grains.

4.2 Artigo 2 – Genome-wide transcriptional profiling of rice unveils novel genes involved in tolerance to iron excess

Artigo com provável submissão à revista *The Plant Genome*

Artur Teixeira de Araujo Junior[1,2]; Daniel da Rosa Farias[2]; Railson Schreinert dos Santos[1,2]; Luis Willian Pacheco Arge[2]; Danyela de Cássia da Silva Oliveira[2]; Solange Ferreira da Silveira Silveira[2]; Camila Pegoraro[2]; Eugenia Jacyra Bolacel Braga[3]; Luciano Carlos da Maia[2]; Antonio Costa de Oliveira[1,2]

[1] Technology Development Center (CDTec), Universidade Federal de Pelotas; Pelotas, RS, Brazil.

[2] Plant Genomics and Breeding Center, Universidade Federal de Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil. Phone: 0555332757263, Fax: 0555332759031, acostol@terra.com.br

[3] Department of Botany, Federal University of Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil

Abstract

Iron (Fe) is essential for plant growth and development. However, high concentrations of Fe(II) in the soil can be toxic for plants. Fe homeostasis is not completely understood and there is little information about molecular responses to excessive amounts of Fe. Rice (*Oryza sativa* L) is a model species for functional genomics and an important crop for human nutrition. Due to the little information available on this subject, this study aimed to investigate iron homeostasis in rice analyzing its expression profile under high concentrations of iron. Here, a tolerant cultivar (BRS Querência), was exposed to high a concentration of iron (300 mg L^{-1} Fe^{+2}). The response of this genotype includes a large increase in the expression of genes, responsible for transport and storage of iron, heat shock proteins and genes related to photosystem II. Conversely, genes associated with synthesis-degradation of ethylene and kinases receptor was down regulated. These results provide novel information about the regulation of genes in an iron tolerant genotype.

Keywords: transcriptomics; iron toxicity; abiotic stress; homeostasis; RNAseq.

1 Introduction

Abiotic and biotic stresses can deeply impact yield. In order to minimize the harmful effects that these stresses can promote plants developed a series of physiological responses (Ohta et al., 2006). Iron (Fe) is considered an essential element for plant growth, however high concentrations of this element can promote toxicity causing severe damage (Mongon et al., 2014; Finatto et al., 2015).

Substantial progress has been made in understanding the molecular aspects of tolerance to iron excess. Three mechanisms are the most important: I) in the root surface through oxidation and precipitation of Fe(II); II) inside the cell through the absorbing of Fe(II) of the extracellular environment and storing in a less reactive form; and III) triggering a tolerance to products formed of reactive oxygen species (ROS) by the Fenton's reaction (Wu et al., 2014). Still most information about iron homeostasis is associated with Fe deficiency and not with its excess (Kobayashi & Nishizawa, 2012).

Rice (*Oryza sativa* L.) is one of the most important staple crops, having a significant importance at economic, social and scientific levels (Finatto et al., 2015). At the economic level, the rice stands out as being the second most widely grown cereal. At the social level, rice is a staple food, present in more than two thirds of the world's population (Finatto et al., 2015). Besides this, rice has a great importance at the scientific level, being considered a model plant among the monocots (Yu et al., 2002).

The process of plant adaptation in stressful situations engage in trigger a series of responses that allows changes in the cell and in the organism so as to maintain optimal cell activities. Understanding how plant cells monitor and respond to external signals in these processes is fundamentally important. The aim of this study was to observe the transcriptional profile of a variety previously described as tolerant to excess iron stress and identify possible stress-responsive genes.

2 Materials and Methods

2.1 Data analysis

The RNA-Seq technique was used to compare the transcriptional profile of rice leaves of a tolerant cultivar (BRS Querência), in stage V3, exposed to iron stresses (300 mg L^{-1} Fe^{+2}) for 24 hours. Data analysis was performed according to Amaral et al. (2016 - Submitted).

The identification of differentially expressed genes (DEGs) was performed using edge R Ver. 3.8.5 (Rensink et al., 2005). The expression levels were normalized by Reads Per Kilobase per Million mapped reads (RPKM) method, with p-value < 0.01. For the enrichment analysis and classification of gene ontology (GO) the BLAST2GO Ver. 2.7.2 was used, the terms of overrepresented GOs were identified by Fisher's exact test (p < 0.01), corrected by the method FDR (false discovery rate) at p < 0.05 (Conesa & Götz, 2008). The annotation was complemented with the database from the Rice Annotation Project Database (RAP-DB: http://rapdb.dna.affrc.go.jp/) (Sakai et al., 2013). After, MapMan Ver. 3.5.1.R2 (Thimm et al., 2004) was used to detect changes in metabolism and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING: http://string-db.org/) to detect the protein-protein interactions with medium confidence (0.4) (Szklarczyk et al., 2015). The representation of the intensity of differential expression was presented in heatmap form by the MultiExperiment Viewer (MeV) Ver. 4.9 (Saeed et al., 2003).

3 Results and Discussion

Differentially expressed genes from RNA-seq data

The transcriptomic profile reports a set of 630 differentially expressed rice genes (DEGs) under iron overload (Additional file 1). These genes represent a small genic fraction (1.37%) of rice genome. Among these DEGs, 334 were up-regulated (53%) and 296 down regulated (47%) (Figure 1A).

Most of these genes (90%) are in range from -1 to 1 Log_2 FC (Fold Change) (Figure 1B). Outside this range, we have a higher number of down regulated genes. The highest fold-changes were found to be -2.67 for Os08g0520000 and 3.594 for Os09g0396900.

The low number of DEGs may be due to the most dramatic changes in transcriptome happening in root epidermis since it is in direct contact with the stress. On the other hand, the

responses were analyzed in a short time under stress, resulting in an initial response front the stress and in the initial transport of the iron for the shoot. Wherein each cell type can be affected in a different form, due the accumulation capacity of the metal in the cell (Morrissey & Guerinot, 2009). Similar data were found by Quinet et al. (2012) that found different response at short- (three days) or long-term (three weeks) and in roots or shoots under iron stress.

The small variation between up and down regulated and the number of DEGs were also found in Quinet et al. (2012). They studied gene expression in cultivar I Kong Pao (*indica* rice), in response to 125 mg L^{-1} $FeSO_4$ for two different times. They obtained 799 different express genes, among them, 64.95% were induced and 35.05% were repressed. The I Kong Pao cultivar was classified as tolerant to iron toxicity just like the BRS Querência.

In opposition, Finatto et al. (2015) found 2525 different express genes, being them 97% were up-regulated. The study used in leaves of 14-day-old rice seedlings (*Oryza sativa* ssp. *japonica* cv. Nipponbare) after put over four days of iron excess exposure (390 mg L^{-1} of Fe^{2+}). This cultivar of rice was considered susceptible (Finatto et al., 2015). This can be suggest that maybe tolerant cultivar can have a more specific response by activating and repressing specific genes associated with tolerance mechanism, whereas in the susceptible plants different metabolisms are altered in an attempt survival.

Gene ontology

Classification of differentially expressed genes for cellular component (Additional file 2) shows great importance of gene products located in mitochondria (96 assignments: 57 up and 39 down regulated), followed by products related to plastids (93 assignments: 54 up and 39 down regulated) and finally to cytoplasmic membrane-bounded vesicle (71 assignments: 23 up and 48 down regulated).

Fe is essential for the reactions present in mitochondria and chloroplasts. The data available in literature indicate that mitochondria are one of the major cellular components affected by some types of stress, mainly because the mitochondria are an important ROS sources and may have a possible role for metal-reductases (Zykova & Kolesnichenko, 2002; Vigani et al., 2013). Some enzymes responsible for antioxidant defenses are present in mitochondria and chloroplasts, having high correlation with the level of Fe. Moreover, Fe may be involved in

the oxidative damage of lipids and proteins (Iturbe-Ormaetxe et al., 1995; Becana et al., 1998).

Concerning the molecular functions, the structural constituent of ribosome, protein binding and ATP binding are the most highly represented in DEGs, with respectively, 30, 16 and 14 assignments. Wherein structural constituent of ribosome, only up regulated genes are observed under iron excess. For down regulated genes we have that the most molecular function are protein binding (seven assignments) and ATP binding (six assignments). Regarding the classification for biological processes, the most frequent differentially expressed genes are related to translation and ribosome biogenesis, where in both cases, we have only up regulated genes, with 27 and 25 assignments, respectively.

The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from the damage (Fulda, Gorman, Hori, & Samali, 2010). To trigger a response mechanism in the plant mostly need to increase the expression of certain genes, having thus, a higher activation of the transcription and translation machinery.

Metabolisms affected by stress iron

Among the different metabolisms affected by stress, we have in featured the iron homeostasis metabolism with a large increase in the expression of genes encoding proteins responsible for transporter and storage of iron (Figure 2), the stress response metabolism with activation of genes that encoding specific heat shock proteins (Figure 3), metabolism of the photosynthesis with a differentiated expression of genes related to photosynthesis (Figure 4), hormone metabolism with the repression of genes associated with ethylene (Figure 5) and the signaling metabolism with repression of genes encoding kinases receptor proteins (Figure 6).

Expression profiles of genes encoding proteins involved in iron homeostasis

Comparing our results with data related to iron metabolism available in literature (Gross et al., 2003; Kobayashi & Nishizawa, 2012; Quinet et al., 2012; Darbani et al., 2013; Bashir et al., 2014; Finatto et al., 2015), we found that 111 genes present in this study are also differentially expressed in others experiments (Additional file 3), may be part of a similar plant response to stress by iron.

Four genes, found in our experiment, are in direct relationship with the central genes responsible for Fe homeostasis and had an increase in the expression (Table 1) (Kobayashi & Nishizawa, 2012). The Os06g0112200 encode a METHYLTHIOADENOSINE NUCLEOSIDASE (MTN) protein that takes part in cysteine and methionine metabolism, present as a regulated enzyme in the methionine (Met) cycle (Bürstenbinder et al., 2010). In dicot plants, the Met cycle produces NICOTIANAMINE (NA) that can have a relationship with metal cation homeostasis (Higuchi et al., 2001; Bürstenbinder et al., 2010). Moreover, this pathway operate NICOTIANAMINE SYNTHASE (NAS), NICOTIANAMINE AMINOTRANSFERASE (NAAT), and DEOXYMUGINEIC ACID SYNTHASE (DMAS) whose products act in the Strategy II of Fe acquisition that is specific to graminaceous plants (Kobayashi & Nishizawa, 2012). The increase of the MTN transcript level is associated with the up regulated ethylene biosynthesis, an important hormone that affects a variety of processes, including the stress response (Bürstenbinder et al., 2010; Wang, Cui, Sun, & Dong, 2013).

The gene Os02g0833100 coding to FERRIC REDUCTASE DEFECTIVE 3 (FRD3), involved in xylem and phloem Fe loading, delivering iron to the shoot in a usable form (Rogers & Guerinot, 2002). Studies suggesting that FRD3 is a transporter of the multidrug and toxin efflux family (Rogers & Guerinot, 2002; Durrett et al., 2007). In rice, most of the data reported the expression of this gene in root pericycle cell, being responsible to regulate citrate effluxes required for efficient Fe translocation (Yokosho et al., 2009). No reports were found on the real function of FRD3 in the leaf. However, the increase of expression in this gene may play an important role as an iron chelator, contributing to iron homeostasis (Rogers & Guerinot, 2002; Durrett, Gassmann, & Rogers, 2007).

We found strong expression of FERRITIN (Os12g0106000), an iron storage protein that acts as an iron buffer being responsible by storing this ion in a soluble, non-toxic and bioavailable form (Petit, Briat, & Lobréaux, 2001). Similar data was found in rice exposed to iron excess showing an increase of the expression of this gene in roots after 3 days of stress under 125 mg L^{-1} $FeSO_4$ (Quinet et al., 2012) and in flag leaves and panicles at different reproductive stages under 9 mM Fe^{2+} (Stein, Ricachenevsky, & Fett, 2009). This gene is associated to plant response to Fe overload, preventing oxidative stress (Petit et al., 2001). The *FERRITIN* was used to biofortification, being that overexpression of this gene results in three fold increases in leaf and seed Fe content, being responsible for storing iron in different tissues (Goto et al.,

1999; Wuytswinkel, V. et al, 1999). This elevation in the expression indicates the participation of this gene in the homeostasis of Fe.

Under the conditions used the gene Os09g0396900, a *VACUOLAR IRON TRANSPORTER (VIT)* gene was induced. Similar data was found in root and shoot rice under iron excess stress (Quinet et al., 2012; Finatto et al., 2015). This gene localizes to the vacuolar membrane and has the function of transporting ions (Fe and Zn) into the vacuole, storing metals under excess conditions and providing in deficiency conditions (Zhang, Y. et al., 2012), being an important mechanism in regulating iron homeostasis (Finatto et al., 2015). Studies suggesting that this gene can be a strategy for producing Fe-biofortified rice, where knockout genes *OsVIT1* or *OsVIT2* can increase the Fe concentration of seeds and decrease in leaves (Zhang, Y. et al., 2012; Bashir et al., 2013). Beyond this gene, we found other metal transporter genes, Os05g0198400, which had its expression affected by stress. Os05g0198400 coding an IRON-REGULATED TRANSPORTER-LIKE PROTEIN 4 (ZIP4) been involved in metal uptake and transport in plants (Ishimaru, 2005). Rice ZIP4 can transport Zn, but no data about transporting other metals, as Fe or Mn have been found (Ramesh et al., 2003; Y. Ishimaru, 2005). However, its increase may be associated with a response to not having a Zn nutritional deficiency in the plant due preventing the uptake, transport and use that iron excess can cause a Fe accumulation on the roots (M. Zhang et al., 2014).

Analyzing the expression profile by Genevestigator (Figure 2A) we found that the *MTN* and *FRD3* have not big differentiation in the expression under nutrient deficiency or submergence condition. However, *FERRITIN* and *VIT1* show a low expression in Fe deficiency condition and with high expression under submergence condition. These data demonstrate that these genes have a higher probability of being expressed in high concentrations of metals to develop a response in cell homeostasis.

The iron homeostasis genes found don't show data of co-expression with the other genes expressed in this experiment. However, products these four genes have co-expression with other proteins (Figure 2B, complete information in Additional file 4). The MTN was the only protein that has data in rice, having a co-expression with a METHYLTHIORIBOSE KINASE (MTK), 0.113, and with ACIREDUCTONE DIOXYGENASE (ARD), 0.142, being that these two proteins make part of Met cycle. The other proteins have co-expression of homologous in different species. The FRD3 has co-expression with an uncharacterized protein, FERRITIN with a 60S ribosomal protein L19 in *Arabidopsis thaliana* and VIT with a transmembrane 9

superfamily member. These data can suggest a possible influence of the iron homeostasis genes in others process of the plant, helping in understand how the stress response can affect the cell.

Specific types of Heat Shock proteins are induced

Genes encode to heat shock proteins (HSPs) exhibited a complex response pattern to iron excess. HSPs are characterized as molecular chaperons (Hu, Hu, & Han, 2009). Studies suggest that the increases of the concentration of HSPs are closely related to resistance to stress (Ray, 1999; Iba, 2002), having an important role in abiotic stress response in plants (Pegoraro et al., 2011). We found nine proteins classified as HSPs, being five up regulated and four down regulated (Figure 3A). Between the HSPs up regulated, two are HSP70, two HSP40 e one is a GrpE. The HSP70 are present in many cellular process, being responsible to protect cells by different types of stress (Samali & Cotter, 1996; Jäättelä et al., 1998), can also make complex with cochaperones, including DnaJ/HSP40 that have too an increase in expression (Takayama, 1997; Qiu et al., 2006). Similar data was found in two *HSPs*, Os12g0569700 and Os03g0271400, that were too down regulated by Fe excess in shoots (Bashir et al., 2014). As can be seen in Figure 3B the expression pattern of these genes are more down regulated under submergence stress conditions, while under nutrient stress, these genes have a higher tendency to be up regulated. These data suggest a more specific response of those genes under different stress conditions, being the expression pattern obtained in this experiment had differences of other conditions analyzed.

As shown in Figure 3C (and Additional file 4), we can observe the network of protein-protein interactions of the HSPs (present in the box denominated 'biotic and abiotic stress') found in this experiment with the other different express genes. These data demonstrate the large amount of positive co-expression data that the HSPs have with other metabolisms induced in this experiment, including mainly oxidases, RNA processing and ribosomal protein synthesis. Similar data were obtained in others studies demonstrating the diverse activities that these proteins may possess, including protein import into organelles (Shi & Theg, 2010), gene regulation by interaction with heat shock transcription factor (HSF) (Kim & Schöffl, 2002), degradation of highly accumulated non-localized proteins (Lee et al., 2009) and protein-folding of de novo-synthesized polypeptides (Hartl, 1996), affecting various metabolisms. Although the functions of HSPs are comprehensively characterized, the

association of the their role in iron excess tolerance has not been investigated thoroughly. Thus, the results obtained in this experiment can help in this process of understanding of plant tolerance response and how these genes are associated with Fe homeostasis

Effects of iron excess on photosynthesis genes

The great majority of genes associated to photosynthesis light reactions were induced in rice under excess iron (Figure 4A and complete information in Additional file 1). Among them highlight the sequences coding for photosystem II (PSII) polypeptide subunits, electron carrier, rubisco large subunit and phosphoglycerate kinase. However, a group of genes of *LIGHT-HARVESTING COMPLEX II* (*LHC-II*) were down regulated (Figure 4A).

In higher plants, LHC-II proteins are mostly associated with PSII, being responsible for absorb photons, effectively deliver and regulate the excitation energy to the reaction centers (Lokstein, Betke, Krikunova, Teuchner, & Voigt, 2012). Between the *LHC-II* genes, we found three genes down regulated related to CHLOROPHYLL A/B-BINDING protein, Os09g0346500 (*OsLhcb1*), Os03g0592500 (*LHCB/LHCP II*) and Os01g0600900 (*OsLhcp*). In contrast, one *Lhcb* gene, *OsLhcb1.3* (Os01g0720500), under excess Fe in rice was up regulated in shoots (Bashir et al., 2014). Although in experiments under Fe deficiency *Lhcb* genes up and down regulated were found (Bashir et al., 2014), demonstrating that the two have different forms of expression may be present. Furthermore, studies suggest that a DnaJ protein, *OsDjA7/8*, has an effect on the expression of *Lhcb* genes in rice (Zhu et al., 2015). Thus, the differential expression of Hsp40 family (Figure 4A and Additional file 1) may contribute for the decrease in the expression of LHC proteins.

Six sequences that code for PSII polypeptide subunits had their highest expressions (Figure 4A). Similar results were found in Bashir et al. (2014) where genes related to PSII were also up regulated while under Fe deficiency these genes were down regulated, suggesting that these genes have an elevated expression, due to increase in rate of photosynthesis provided by the high availability of Fe (Bashir et al., 2014). In addition, changes in the expression of these genes have being found in other studies under the stress condition (Pegoraro et al., 2015), despite that, according to the analysis in Genevestigator (Figure 4B), we until have a little data on this expression, being specific to some genes of this family. Besides this, we also found in the literature other genes associated with light reaction that also were increased

under iron excess (Figure 4A). These genes may be associated with a response similar of the plant to this stress condition (Bashir et al., 2014; Finatto et al., 2015).

The co-expression data in rice shows that the genes differentially expressed associated to photosynthesis has interactions with nine metabolisms (Figure 4C). Although it has few proteins associated with some metabolism (redox and regulation of transcription), these may contribute to important associations. The interaction with redox metabolism is due the gene encoding the ascorbate, an antioxidant and a co-factor of many metal-containing enzymes, that has multiple roles in stress tolerance and in plant development (Davey et al., 2000). In the photosynthesis, ascorbate can have different functions as an alternative electron donor for PSI and PSII, and can be an antioxidant in chloroplasts (Mano, Hideg, & Asada, 2004; Höller et al., 2015). In the regulation of transcription we have a Heat-shock transcription factor family, whereupon, already mentioned the great importance of this class in the stress response.

The majority of the genes associated with hormone response are down regulated

We found changes in expression of genes that are involved in the biosynthesis of plant hormones (Additional file 1). Between these genes, five are associates to synthesis-degradation of ethylene (Figure 5A), six to abscisic acid (ABA) signaling pathway and four to auxin response.

All the genes relative to synthesis-degradation of ethylene were down regulated, this may be due to decreased expression of two ethylene responsive factors, Os09g0287000 (*OsERF63*) and Os09g0434500 (*OsERF72*). The AP2/ETHYLENE RESPONSE FACTOR (ERF) can act as a transcriptional activator and as repressor activity, being that these genes can generate tolerance (Thirugnanasambantham et al., 2015). As can be seen in Figure 5B, these sequences that code for synthesis-degradation of ethylene have been identified having changes in their expression profile of stress. Finatto et al. (2015) found up regulation in genes associated to ethylene response in rice under iron excess. However, their cultivar was characterized having a sensitivity to stress, moreover it has not found the same set of genes associated with the ethylene response.

The expression of others genes associated with the plant hormones also were down regulated. The genes relatives to abscisic acid had a decrease in expression, this can be explained due the also descrease in ABA-activated protein kinase, Os02g0551100 and Os04g0691100. Besides

this, the majority of the auxin genes were down regulated. These hormone response suggesting that the associated to ethylene response, ABA signaling and auxin response may be involved in Fe excess stress responses, resulting in a wide reprogramming development mediated by hormones in response to stress.

The co-expression data show a few numbers of interactions presents in three metabolisms and a group of unknown (Figure 5C). Hormone metabolism have interactions with genes associated with auxin and ethylene, moreover, have interactions with different class of factor family in the metabolism related with regulation of transcription, suggesting that the hormone metabolism may have a complex regulation or contribute to the expression of several factors. These results may contribute to the understanding of the hormonal mechanism and how this mechanism may act developing a response to stress.

Receptor kinases are down regulated in conditions of iron excess

The plant receptor protein kinase consists of transmembrane proteins that perceive stimuli extracellular and transmits response signals to these stimuli, being associated with trigger to abiotic and biotic stress response (Shiu & Bleecker, 2001; Morris & Walker, 2003). All the differential express sequences that coding to receptor protein kinase were down regulated under iron excess (Figure 6A).

This may trigger a specific type of response to this stress, being characteristic of this cultivar. The expression profile of these genes on different stresses (Figure 6B) indicates that many of these genes do not have a differential expression in the conditions analyzed. However two genes, Os07g0628900 and Os07g0628700, have an expression in up and down regulated. These observations suggest that the decrease on the expression of these genes may directly effect on generation of compounds and thus may promoting a differentiated response.

Analyzing the co-expression of this metabolism we have the interaction of a few numbers of proteins that make part of six known metabolisms (Figure 6C). Among those metabolisms we have in highlighted the oxidases, glycolysis, photosynthesis and hormone metabolism, being that all these interactions were with sequences down regulated in this study. This result may suggest that differential expression of these signaling genes can be strongly associated with the expression of different groups of proteins, indicating a reprogramming in different cell metabolism in response to stress.

4 Conclusion

Plants have evolved a myriad of adaptive mechanisms based on a number of genes to deal with the different toxic metals they encounter in soils worldwide. In this paper, we identified a large number of up regulated genes involved in iron homeostasis, specific heat shock proteins and photosystem II polypeptide subunits. Furthermore, we found a group of genes associates to ethylene kinases receptor and light-harvesting complex II that were down regulated. These results clarified some response of iron stress in rice plants.

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Additional files

The additional files of this article are present in the Annexes of the dissertation.

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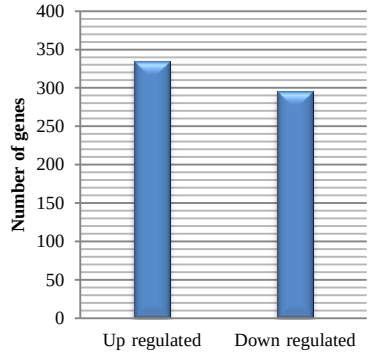
A



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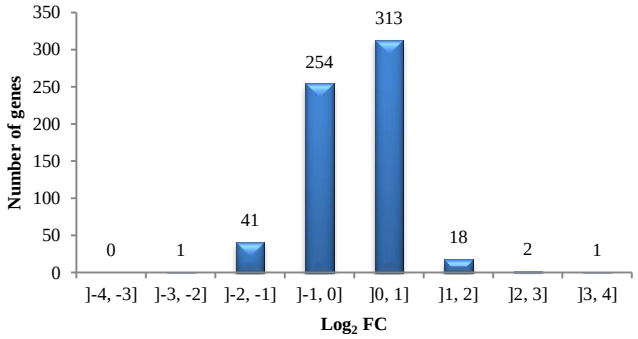

Figure 1. Differentially expressed genes in rice (*Oryza sativa* ssp. *indica* cultivar BRS Querência) under iron excess. A) Number of up and down regulated genes; B) Distribution of frequencies (p-value ≤ 0.01).

Table 1. List of differentially genes express found in the experiment responsible for Fe homeostasis.

Gene-ID	Name	Function	log_2FC
Strategy II Fe uptake			
Os06g0112200	*MTN*	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	0.403
Fe translocation			
Os02g0833100	*FRD3*	Citrate efflux transporter	0.461
Fe storage			
Os12g0106000	*FERRITIN 2*	High-capacity Fe storage and sequestration	2.174
Fe compartmentalization			
Os09g0396900	*VIT1*	Fe transporter into vacuole	3.594

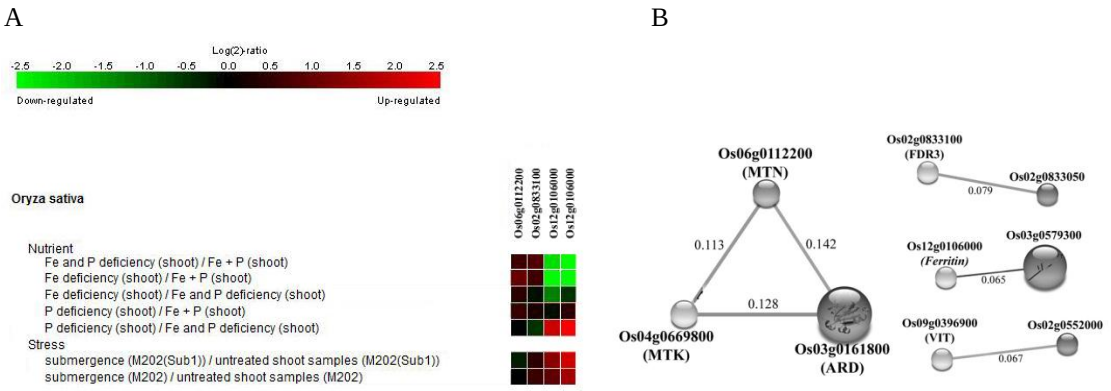

Figure 2. Analysis in the database of differentially expressed genes involved in iron homeostasis. A) Expression analysis in shoot under different stress conditions, nutritional deficiency (iron (Fe) and/or phosphorus (P)) and submergence, provided by Genevestigator. B) Representation of the Protein-Protein Interactions (PPI) network provided by STRING. Have not data about PPI of these four genes with the differentially expressed in this experiment. These being interactions with genes not found in the experiment, where the intensity of the co-expression is demonstrate between the proteins. ACIREDUCTONE DIOXYGENASE (ARD); METHYLTHIORIBOSE KINASE (MTK); 5'-METHYLTHIOADENOSINE NUCLEOSIDASE (MTN); FERRIC REDUCTASE DEFECTIVE 3 (FRD3) AND VACUOLAR IRON TRANSPORTER (VIT).

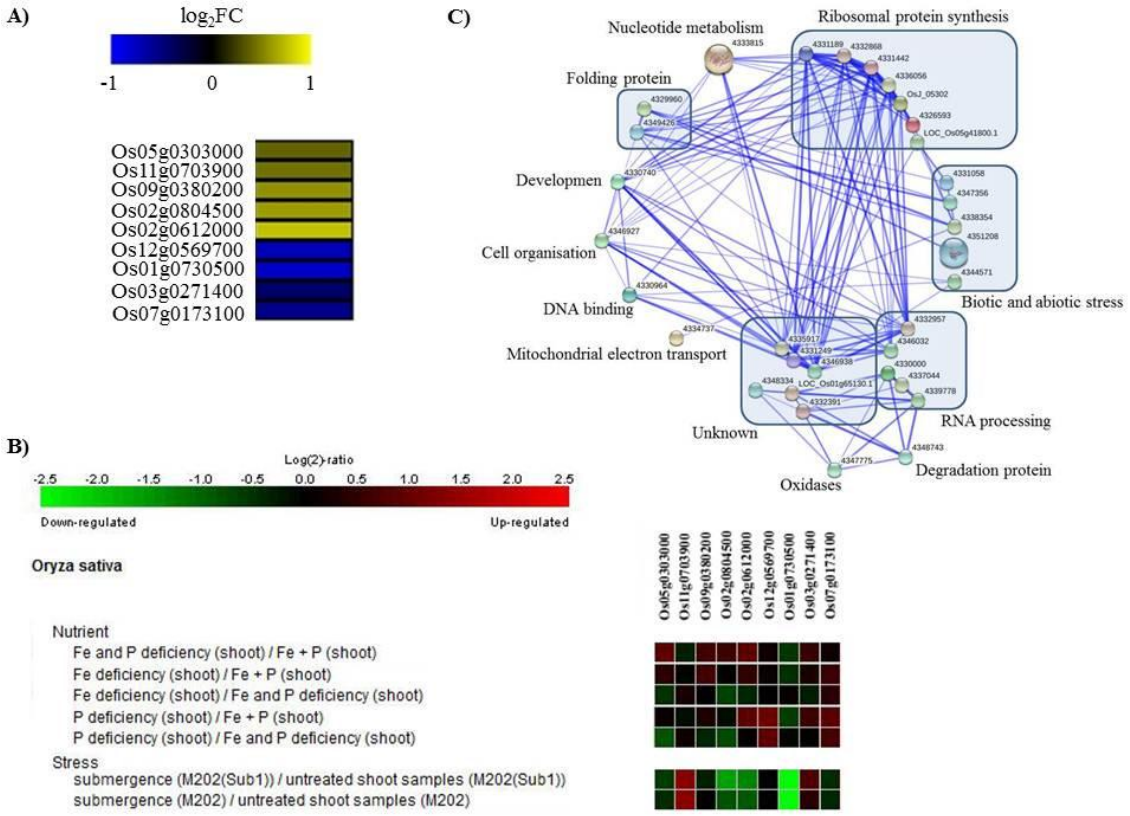

Figure 3. Effects of iron excess on genes expression associate Heat Shock proteins (HSPs). A) Pattern of expression of HSPs genes differentially express under iron excess, data are expressed as a heat map showing the signal intensity. B) Expression analysis in shoot under different stress conditions, nutritional deficiency (iron (Fe) and/or phosphorus (P)) and submergence, provided by Genevestigator. C) Representation of the Protein-Protein Interactions (PPI) network provided by STRING.

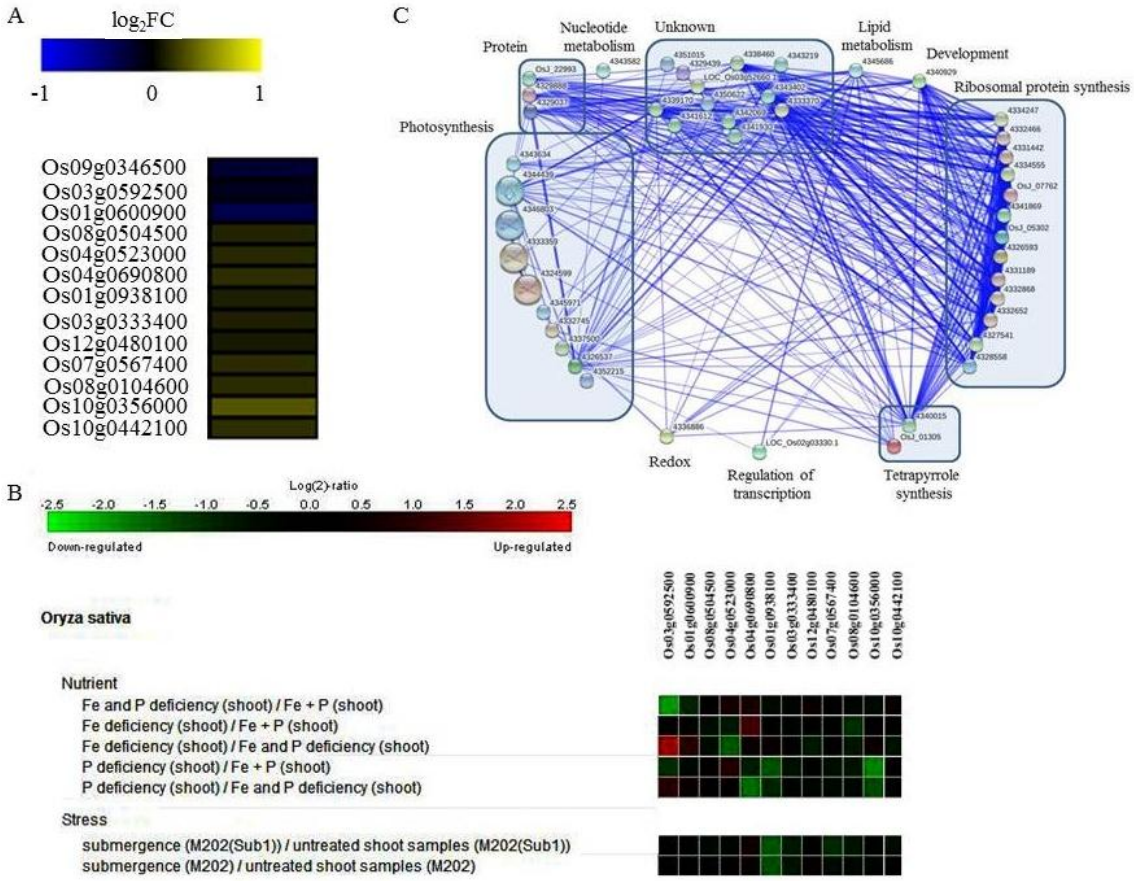

Figure 4. Effects of iron excess on photosynthesis. A) Pattern of expression of genes related to photosynthesis, data are expressed as a heat map showing the signal intensity. B) Expression analysis in shoot under different stress conditions, nutritional deficiency (iron (Fe) and/or phosphorus (P)) and submergence, provided by Genevestigator. C) Representation of the Protein-Protein Interactions (PPI) network provided by STRING.

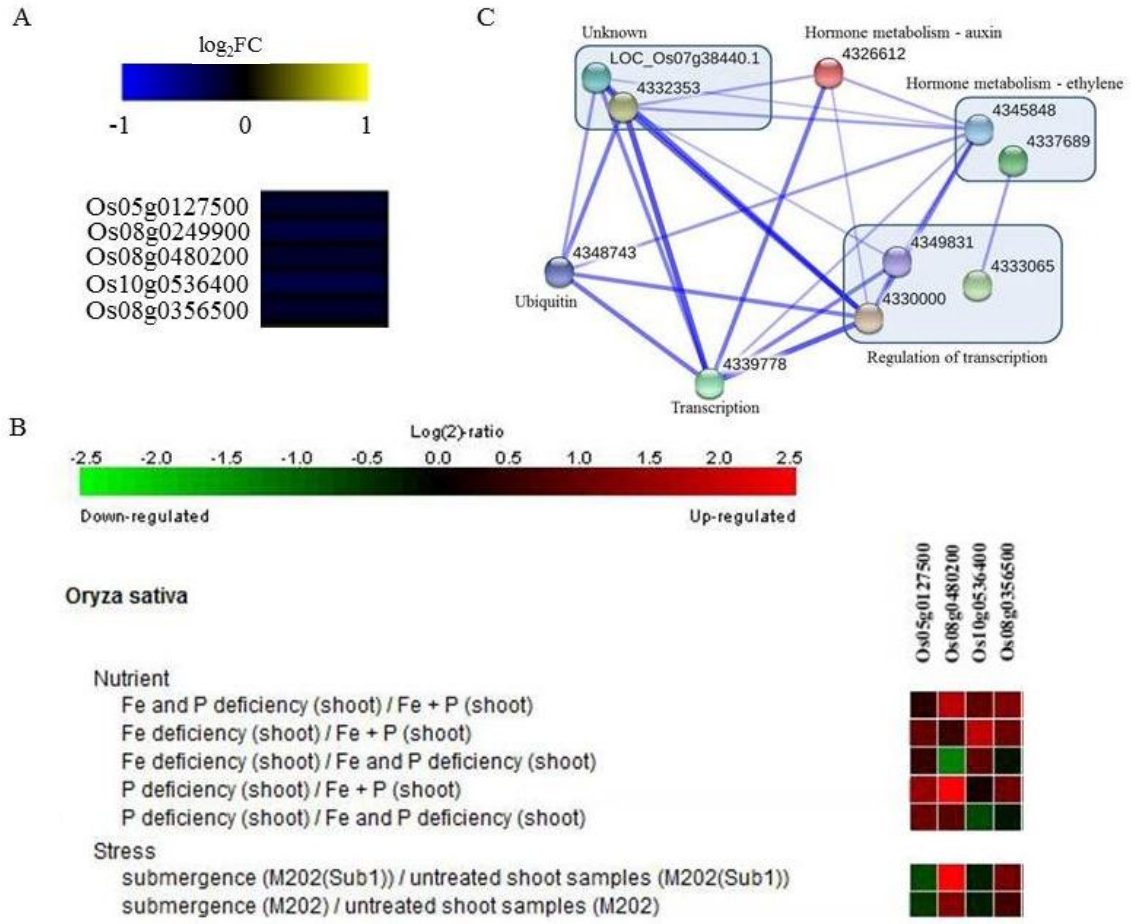

Figure 5. Effects of iron excess on genes expression associate to synthesis-degradation of ethylene. A) Pattern of expression of genes differentially express associate to synthesis-degradation of ethylene under iron excess, data are expressed as a heat map showing the signal intensity. B) Expression analysis in shoot under different stress conditions, nutritional deficiency (iron (Fe) and/or phosphorus (P)) and submergence, provided by Genevestigator. C) Representation of the Protein-Protein Interactions (PPI) network provided by STRING.

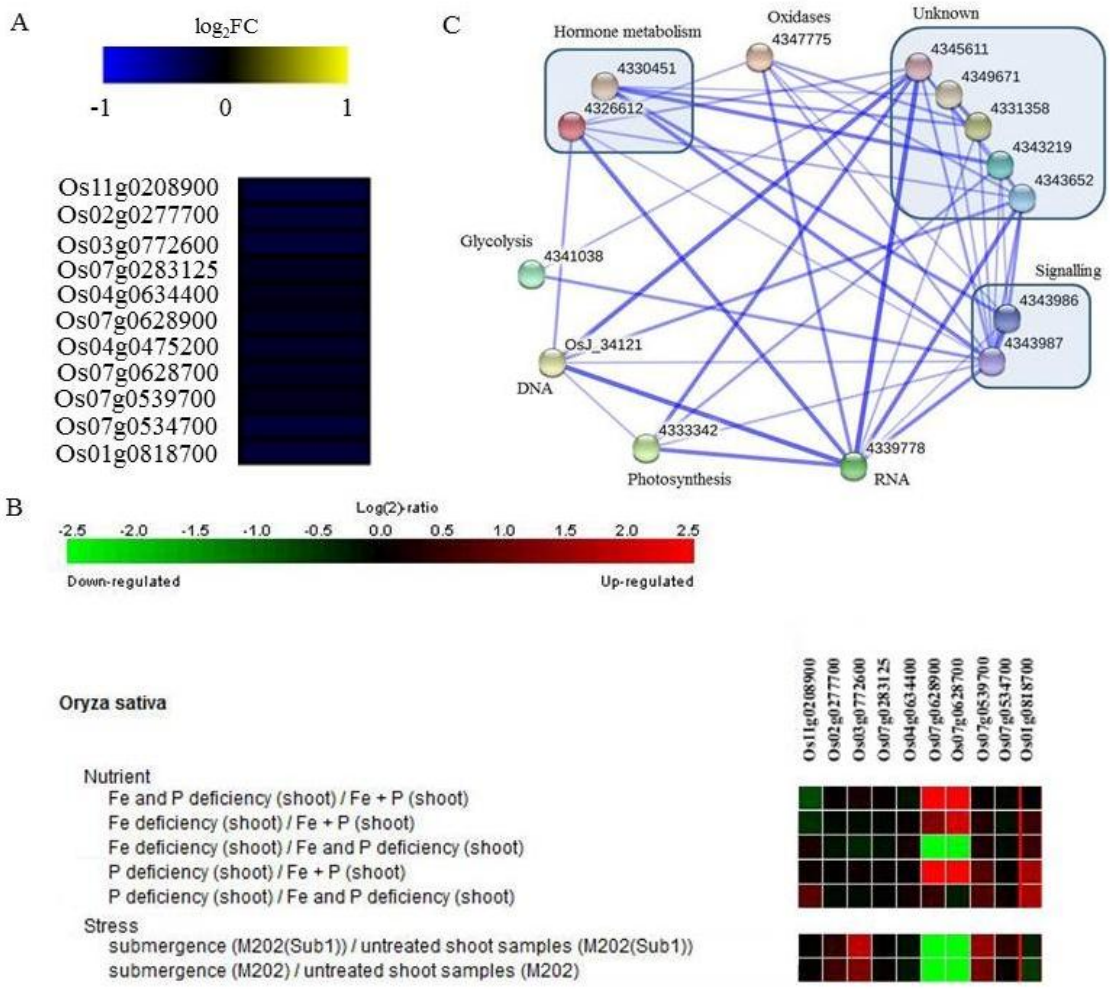

Figure 6. Effects of iron excess on genes expression associate receptor kinases. A) Pattern of expression of genes differentially express associate receptor kinases under iron excess, data are expressed as a heat map showing the signal intensity. B) Expression analysis in shoot under different stress conditions, nutritional deficiency (iron (Fe) and/or phosphorus (P)) and submergence, provided by Genevestigator. C) Representation of the Protein-Protein Interactions (PPI) network provided by STRING.

4.3 Artigo 3 – Stress response of plants: the role of alternative splicing

Artigo com provável submissão à revista *Genome*

Artur Teixeira de Araujo Junior[1,2]; Railson Schreinert dos Santos[1,2]; Daniel da Rosa Farias[2]; Danyela de Cássia da Silva Oliveira[2]; Solange Ferreira da Silveira Silveira[2]; Camila Pegoraro[2]; Luciano Carlos da Maia[2]; Antonio Costa de Oliveira[1,2*]

[1]Technology Development Center (CDTec), Federal University of Pelotas; Pelotas, RS, Brazil

[2]Plant Genomics and Breeding Center, Federal University of Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil.

* Corresponding author: Antonio Costa de Oliveira; Plant Genomics and Breeding Center, Universidade Federal de Pelotas; PO Box 354, Campus Universitário, S/N - CEP 96160-000, Capão do Leão, RS, Brazil.
E-mail: acostol@cgfufpel.org

Abstract

In course of time, plants have developed mechanisms to cope and adapt to different types of stresses (abiotic and biotic) frequently imposed by the adverse environment. These conditions can induce a range of morphological, physiological, biochemical and molecular responses in plants. Advances in molecular analyses showed new evidence that alternative splicing (AS) is a major mechanism responsible for the enhancement of transcriptomic and proteomic diversity, having different roles in plant development and stress tolerance. In this review, we discuss regulation, types and consequences of alternative splicing and the relationship between changes in the AS and the stress tolerance in plants.

Keywords: biotic stress, abiotic stress, resistance

Introduction

Recent studies show that the condition of biotic (insects, pathogens, weed competition) and abiotic stresses (heat, cold, heavy metals and others) can affect the alternative splicing (AS) of pre-mRNAs in plants (Dinesh-Kumar & Baker 2000; Reddy 2001; Jordan et al. 2002). These findings suggest that splice variants are affected by stresses conditions (Reddy 2007). This raised many questions about the relationship between AS and stress tolerance in plants.

AS is prevalent in eukaryotic organisms, affecting nearly 95% of mammalian genes (Pan et al. 2008; Barash et al. 2010), however, some cases of splicing have been reported in bacteria (Edgell et al. 2000) and archea (Watanabe et al. 2002; Yokobori et al. 2009). AS is capable of creating multiple mRNA transcripts from a single gene (Barbazuk et al. 2008). These new proteins isoforms can lose or gain new functions and alter their cellular localization, stability and activity (Kelemen et al. 2013). Being important for regulation of the levels and tissue specificity of gene expression (Wang & Cooper 2007; Tazi et al. 2009).

In plants, it has been discovered that at least 30% of the genes undergo AS, which evidences its significance to transcriptome and proteome complexity (Wang & Brendel 2006; Compans & Cooper 2008).

The interest in the relationship between AS and the different types of stress has increased (Bassett 1989). New data has helped the understanding about how new isoforms can influence

the survival of the organism and how the regulation of AS is performed. Thus, we need to group these new results to better understand how these increases in diversity of proteins helps to maintain the complexity of life under different stress conditions.

In this Review, regulation, types and consequences of AS and the relationship between AS and the possible increase of tolerance in plants is discussed, focused on understanding the role of alternative splicing in plant responses to environmental stresses. We start by discussing mechanisms of AS. After, we evaluated the changes that are observed when plants are under stress and how these affect AS.

Regulation, types and consequences of alternative splicing

In 1977, the first noncoding sequences in the Adenovirus *hexon* gene was discovered, after that, others studies found that most eukaryotic genes contain noncoding intervening sequences (introns), promoting new research to understand the role of these sequences in the genomes (Berget et al. 1977; Chow et al. 1977). In the mid 80s, we started to use the terminology "alternative splicing" to describe multiple mRNAs produced by the human growth hormone (hgH) gene, demonstrating that introns can expand the diversity of proteins (Denoto et al. 1981). Since then, the number of papers involving AS increased over time (Figure 1).

In bacteria and archea, AS is considered to be rare, with just a few examples, as in genes encoding a homolog of eukaryotic Cbf5p in some species of archea and in bacteria with different groups of introns of *thymidylate synthase* (*td*) gene (Edgell et al. 2000; Watanabe et al. 2002; Yokobori et al. 2009). Eukaryotes have a much higher frequency of AS in multicellular than in unicellular organisms (Liang et al. 2003), being reported in three genes of the budding yeast *Saccharomyces cerevisiae* (Davis et al. 2000; Graveley 2001), hemiascomycete *Yarrowia lipolytica* (Neuvéglise et al. 2005), *Schizosaccharomyces pombe* (Okazaki & Niwa 2000) and the malaria parasite *Plasmodium falciparum* (Muhia et al. 2003). Among the multicellular organisms, AS seems to be especially important for vertebrates, due to the high complexity of these organisms (Kornblihtt et al. 2013).

The percentage of studies about plant AS is very low when compared to the whole data on this topic (Figure 1). However, recent research on plant genomes (Feng et al. 2002; Sasaki et al. 2002) and characterization of spliceosomal proteins (Reddy 2001), has enabled an increase

in the number of articles on this topic, thus being able to explore AS events that happen in a particular plant and compare with existing data (Wang & Brendel 2006).

There are different types of AS, including exon skipping (ExonS), alternative donor (AltD) or acceptor (AltA) site, intron retention (IntronR), and others (Wang & Brendel 2006). These different types can be regulated at many different levels (Keren et al. 2010), transform this event in an important role in development and differentiation of multicellular organisms and in producing structurally and functionally different proteins from a single gene (Smith & Valcárcel 2000; Jorcano & Río 2011).

Insights in regulation of the AS

One of the challenges to the splicing machinery (spliceosome) is to recognize and distinct RNA sequences across the transcriptome and appropriately regulate AS to meet the physiological requirements of cells and tissues. Part of these challenges consist in several intercommunicating layers of *cis*-acting elements that auxiliary the AS events and serve as binding sites for auxiliary factors that regulate alternative splicing (Wang & Cooper 2007). This regulation suggests the existence of a 'splicing code' and its understanding should be important to comprehend and predict the mechanism of response of the organism under some conditions (Fu & Ares 2014).

Recent studies have demonstrated that there is an elaborate crosstalk among multiple layers of posttranscriptional regulation and between transcription and chromatin. This evidence suggest that the AS can be regulated by the interaction between chromatin landscape changes, such as nucleosome positioning, histone marks, and DNA methylation, together with RNA structural features and splicing regulatory elements (SREs) (Reddy et al. 2013).

An important part of the regulation is promoted by some specific sequences and factors (Kornblihtt et al. 2013). These short (5 to 10 nucleotides) conserved sequences are denominated as *cis*-acting elements present in exons and introns, and often occur in clusters (Blencowe 2006; Chen & Manley 2009). These *cis*-regulatory sequences include exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs) (Figure 2), being important for the correct recognition of pairs of splice sites (Chen & Manley 2009; Mcmanus & Graveley 2011).

Studies show that the sequences flanking the splice sites are less conserved in some organisms, requiring additional regulatory sequences adjacent, generally called splicing enhancers/repressors (Graveley 2000; Reddy 2004).

The factors that act in regulating are called *trans*-acting factors and include two main families of AS regulatory proteins, Ser/Arg-rich proteins (SRs) and heterogeneous nuclear ribonucleoprotein (hnRNP), besides, it can include other tissue-specific factors such as neural polypyrimidine tract binding proteins (nPTB) and polypyrimidine tract binding proteins (PTB) (Kafasla et al. 2012), neuro-oncological ventral antigen (NOVA) (Jelen et al. 2007) and forkhead box (FOX) (Lee et al. 2009). These splicing regulatory proteins act by binding with the *cis*-acting elements recruiting/stabilizing spliceosomal components and/or antagonizing other splicing inhibitory proteins, thereby regulating the constitutive splicing (CS) and AS (Reddy et al. 2013).

The hnRNP is characterized for blocking the binding site for splicing factors (Wang & Brendel 2004), being responsible for regulators of diverse processing events including mRNA splicing, transport and stability. Due the multiple functions the same protein can effect regulated RNA processing at more than one level (Krecic & Swanson 1999). These proteins are widely studied, more than 20 abundant hnRNPs were found in human cells (Krecic & Swanson 1999), 12 major proteins in *Drosophila melanogaster* (Haynes et al. 1990; Haynes et al. 1991; Matunis et al. 1993) and 35 potential hnRNP proteins possibly related to splicing was found in *Arabidopsis* (Lambermon et al. 2000).

In plants, the numbers of SR genes are higher than in other organisms, while the sequences are highly conserved. Studies suggesting that there are 11 genes encoding SR proteins in human and seven in *Caenorhabditis elegans*, although, there are 19 in *Arabidopsis* and 24 in rice (Kalyna & Barta 2004; Lida et al. 2004; Isshiki et al. 2006; Reddy 2007). SR protein kinase modulates both constitutive and alternative splicing (Stojdl & Bell 1999), being that different SR proteins can bind to distinct enhancer/silencer sequences and the preference of one may be responsible for causing changes in the morphology of the plant (Savaldi-Goldstein 2003).

Evidences have demonstrated that another part of the regulation can be promoted by chromatin, having a key role in AS through the effects of both histone modifications and nucleosome positioning (Allo et al. 2010; Luco et al. 2011). This could be due the nature of histone marks that are deposited on the chromatin around a gene affected the AS decisions

(Kornblihtt et al. 2013). Between the mechanisms, chromatin can influence alternative splicing by two major ways: neuron depolarization and neuron differentiation, whereupon, can promoted the skipping or inclusion of determinates exons due the increase or reduction in RNA polymerase II (Pol II)-mediated elongation. The modifications that histone can promote are due to the recruitment coupling mechanism (Luco et al. 2010).

Although, the mechanism that control the AS has not been elucidated, this mechanism can be more complex having marked exon-intron boundaries due to DNA modifications, such as cytosine methylation, chromatin changes, histone modifications and nucleosome occupancy (Braunschweig et al. 2013).

In addition, many other factors may affect splicing, either directly or indirectly. Hormones, such as abscisic acid (ABA) and indole-3-acetic acid (IAA), can change the alternative splicing pattern in three SR genes in *Arabidopsis* (Palusa et al. 2007). Ethylene can induce the expression of SR protein kinase PK12 in tobacco (Savaldi-Goldstein et al. 2000). Furthermore, environmental factors, such as temperature, and salinity, have dramatic effects on the alternative splicing pattern of several SR genes (Palusa et al. 2007).

Types of Alternative splicing events

A multiexon gene can produce different mature mRNAs due the multiple possible combinations of these (Reddy et al. 2013). Splicing events can be grouped into four types: exon skipping, alternative 3’ splice site (3’SS) and 5’ splice site (5’SS) selection, and intron retention (Figure 3). Also there are complex types of AS events, which are less frequent, these events include mutually exclusive exons, alternative promoter usage and alternative polyadenylation (Black 2003; Ast 2004; Kim et al. 2008).

In higher eukaryotes, approximately 40% of the AS events are due to exon skipping, followed by alternative 3′SS selection (18.4%) and alternative 5′SS selection (7.9%) (Sugnet et al. 2004; Alekseyenko et al. 2007). Intron retention is relatively rare in vertebrates and invertebrates, constituting less than 5% of the AS events, but in plants, fungi and protozoa this event is the most prevalent, with approximately 30% in plants (Alekseyenko et al. 2007; Kim et al. 2008). These studies suggest that the frequency of each type of AS extremely dependent on the species, organ, type of tissue, cell, and on environmental conditions (Figure 4) (Pan et al. 2008; Wang et al. 2008; Merkin et al. 2012).

The different kinds of AS events can make changes in the functionality of the transcription product, being that part of the exon skipping and alternative 5' or alternative 3' splice sites can produce easier changes in the subcellular localization, binding properties, and activity or stability, while part of the intron retention can result in the production of truncated proteins (Barbazuk et al. 2008; Mastrangelo et al. 2012).

This difference in the proportion of AS types suggests a possible difference in the mechanism of splice site recognition. Studies demonstrated that the splicing mechanisms are closely tied to gene structure (Mcguire et al. 2008). Moreover, the differences in splicing regulatory components also influence the splicing mechanisms (Barbazuk et al. 2008).

Consequences of the AS

The possible effects of alternative splicing on proteins can include distinct protein isoforms with altered (related, distinct, or opposing) functions or can even determine the fate of the transcript trough the regulation of mRNA stability by nonsense-mediated decay (NMD) - and microRNA (miRNA) - mediated mechanisms, or influencing mRNA transport, localization, and translational efficiency (Reddy et al. 2013).

The creation of distinct protein isoforms can increase the proteome diversity. Some AS events can produce truncated protein isoforms. These proteins might still have functional modules, might lack certain functional domains, or lose/gain sites for posttranslational modifications (Reddy et al. 2013). Several splicing variants produce small interfering peptides, which can compete with functional dimers, resulting in dominant-negative regulations (Staudt & Wenkel 2011; Seo et al. 2013).

NMD is a cytoplasmic RNA degradation system, responsible for recruiting mRNA decay enzymes that prematurely terminate the mRNAs. AS can introduce or change the features of NMD substrates altering the stability of a transcript and consequently affecting the transcript abundance. Besides this, AS can modulate miRNA-mediated regulation of gene expression, producing proteins isoforms that contain or lack target sites for miRNA. Furthermore, it is responsible for modulating the levels of miRNA, whereupon is accomplished by regulation the splicing pattern of pri-miRNAs or pre-mRNAs enconding enzymes (Reddy et al. 2013).

Another observation is in the evolution and divergence of the species. Recent studies suggest that AS is important for of organ divergence, and that it may have contributed to speciation (Barbosa-Morais et al. 2012; Merkin et al. 2012). Moreover, studies found identical exon

skipping events in species that diverged more than one billion years ago (Awan et al. 2013). Therewith, tissue-specific gene expression is mostly conserved, whereas AS diverged (Merkin et al. 2012) and the split gene organization could accelerate evolution (Gilbert, 1978).

Changes in alternative splicing by stress

To survive, plants need to adapt and respond to different environmental conditions due to their sessile condition (Kornblihtt et al. 2013), wherein AS is one of the regulatory mechanisms responsible for this response (Barbazuk et al. 2008). The first AS in plants was identified in spinach and *Arabidopsis* in the characterization of *ribulose bisphosphate carboxylase/oxygenase* (rubisco) activase (Reddy 2001). After that, studies regarding AS in plants increased, part of this studies indicate that several biotic and abiotic stresses influence splicing decisions (Zhang & Gassmann 2007; Nakaminami et al. 2012), being considerable an important part of gene regulation in stress responses (Compans & Cooper 2008).

Alternative Splicing in abiotic stress

Abiotic stresses such as drought, salinity and extreme temperatures can limit agricultural productivity. Significant progresses have been done to understand the mechanisms of abiotic stress tolerance in plants (Zhang & Gassmann 2007; Compans & Cooper 2008; Nakaminami et al. 2012). These studies show that abiotic stress promotes changes in gene expression, part of this due to the transcription factors and potential signaling components, that show the occurrence of alternative splicing (Zhu 2002; Yamaguchi-Shinozaki & Shinozaki 2006).

Different stresses can affect the AS of different genes and it also interesting to highlight that some of these genes are affected by multiple stresses (Compans & Cooper 2008). Part of these genes are characterized as post-transcriptional regulation, transcriptional regulation, protein involved in lipid and sugar metabolism and reactive oxygen species (ROS) control.

The concentration of different SR proteins may be altered, being able to change the AS of downstream target genes (Compans & Cooper 2008). *Arabidopsis* submitted to conditions of cold and heat showed a change in the AS patterns, having distinct changes of SR proteins concentration in each type of stress generating different plant responses (Lazar & Goodman 2000; Palusa et al. 2007).

Yeast cells and transgenic *Arabidopsis* showed tolerance to salt when began to express the RS domain of two different SR-like proteins (Forment et al. 2002). Besides, in others studies changes promoted by higher salt levels altered the expression of some SR proteins (SR34b, SR33, and SR30) (Palusa et al. 2007; Tanabe et al. 2007).

The same retention of the fourth intron in RING (Really Interesting New Gene) - finger (6G2) gene was found in *Arabidopsis* under drought and cold stress condition. This may suggest that there is a common mechanism in both stresses, that can regulate this process (Mastrangelo et al. 2005). Others stresses that caused the same changes were intense light stress that altered the splicing pattern of SR30 (Tanabe et al. 2007).

The dehydration responsive element-binding factor 2 (DREB2) is characterized as a transcription factor. This gene in wheat produces three isoforms due the inclusion/exclusion of exons (Xue & Loveridge 2004; Egawa et al. 2006). Studies show that when wheat is subjected to prolonged cold treatment, all of its isoforms have an increase of expression (Compans & Cooper 2008). Similar data was found for salt stress, while in drought stress two isoforms increased the expression, whereas the other isoform remained constant (Xue & Loveridge 2004; Egawa et al. 2006). In maize this gene has only two isoforms, *ZmDREB2A-L* and *ZmDREB2A-S*, that have a preferentially induced by cold and repressed by heat stress (Qin et al. 2007).

The mitogen-activated protein kinase (MAPK) is characterized as universal signaling modules, being involved in many plant processes, including in environmental stress response (Jonak 2002). Studies found five splice variants of MAPK in *Arabidopsis* (Lin et al. 2010). Between these proteins, was identified that the MAPK 13 gene can generate at least three different variants. Wherein, two of these proteins although do not show kinase activity and neither interacted with upstream MAPKKs, one variant protein showed an enhanced the MKK6-dependent activation (Lin et al. 2010). In rice also was found change in one member of the MAPK family, OsBWMK1, that under stress conditions was able to produce splice variants with different subcelular localizations (Koo et al. 2007).

Studies with cold stress have demonstrated a differential ensemble of protein isoforms. In lipid metabolism, the gene *β-hydroxyacyl ACP dehydratase*, which is an enzyme involved in fatty acid biosynthesis, was alternatively spliced in *Picea mariana* (Tai et al. 2006). Another gene, acetyl CoA carboxylase (ACC), which is involved in this metabolism also display an

AS and promoter initiation in both metazoans and plants (Podkowinski 2003; Barber et al. 2005).

Furthermore, in the cold stress, studies have shown a different regulation under this conditions for sugar metabolism, suggesting that these genes can play an essential role. Two early cold-regulated (e-cor) genes that code for a ribokinase (7H8) and a RING-finger protein (6G2) have an increase in isoforms that retained alternative introns, which can result in a nonfunctional truncated protein and in a decrease of the functional proteins (Mastrangelo et al. 2005).

In maize (*Zea mays* L.) two transcription isoforms of *ZmrbohB*, a gene involved in ROS control (Lin et al. 2009). The ROS have an important role in biotic interactions and abiotic stress (Sagi & Fluhr 2006). These splice variants have the expression dependent of the tissue and the development stages, having an increase of expression under biotic and abiotic stress (Lin et al. 2009).

In summary, an increasing number of genes that generate splice variants as a response to environmental stress have been identified. These new data have provided new discoveries about the effect of AS and the expression regulation of plant gene under abiotic stress, helping in understanding the consequences that the change of the pattern AS can cause the plant.

Alternative splicing in biotic stress

An important resource for plant defense against pathogens is the capacity of changing the expression profile of different genes (Wang & Brendel 2006). Studies show that AS has a relevant role in this process, being responsible for the creation of different isoforms of resistance genes (Compans & Cooper 2008).

Among these genes, the highly specific resistance (*R*) genes perform a significant role in plant defense perception of pathogen avirulence (*avr*) genes (Flor 1971). Several reports describe that AS in pre-mRNAs of *R* genes is dynamic during the defense response, therefore, responsible for accomplish an important response of the plant against the pathogens. As a consequence the alternative transcripts can change the expression level of R proteins and produce truncated *R* proteins (Compans & Cooper 2008).

The CC-NBS-LRR (CNL) R genes are characterized for having diverse gene structures. Differences in the regulation of AS is reported in different plant species. In maize it was detected that transcript variants with an intronless open reading frame (ORF) of the *Rp1D*

gene. However, in *Arabidopsis* no report of this type of AS event has been made (Ayliffe et al. 2004).

The CNL genes have been much studied in barley *Mla* genes that control ETI to the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (Bgh). The *Mla13* can produce five transcription variants. Studies suggest that this difference could be responsible for determining its efficiency of translation (Halterman 2003).

Another classes of *R* genes are the members of the TIR-NBS-LRR (TNL) family. Whereupon, diverse cases of AS have been described, including alternative exons, alternative 5' or 3' splice site selection and intron retention. The splicing variants are characterized as being similar proteins and consist of truncated TIR-NBS (TN) proteins (Whitham et al. 1994). Studies suggest that these truncated proteins perform an important function in defense response (Compans & Cooper 2008).

These truncated proteins were reported in the tobacco *N* gene, which specifies resistance to tobacco mosaic virus (TMV). In order to demonstrate full resistance to TMV the presence of only full-length N protein is not enough (Whitham et al. 1994). In *Arabidopsis*, it was found that only one transcription variant of the Resistance to the *Pseudomonas Syringae4 (RPS4)* gene was up regulated during the defense response, the multiple products of these transcripts may have distinct functions in plant response (Zhang & Gassmann 2007).

Combining the information available for these genes can help to elucidate part of the role of alternative splicing under stress conditions (Figure 5). Although, a complete understand of the signaling pathways which are related to stress is still needed.

As shown, stress can regulate splicing, changing multiple mechanisms, including splicing factors, the level of expression of SR proteins or phosphorylation. However, a continued investigation to elucidate the mechanisms of stress response in plants is still necessary, helping in breeding programs.

Conclusion

Studies of alternative splicing in plants have an important contribution for the understanding of the mechanisms of regulation of these process and consequently plant response under different stress conditions. These studies have helped to elucidate the transcriptome and proteome complexity and how the tolerance response is achieved, being an important step in

obtaining improved varieties. Here we showed how the alternative splicing occur, their main means of regulation and some of its consequences. Demonstration, how the alternative splicing is a powerful mechanism that can be used for selection of specific events associated to some splice variants that can increase stress resistance. However, more studies on the consequences of these new variants to become a good strategy, particularly for their actual usage in scenario of climate change are necessary.

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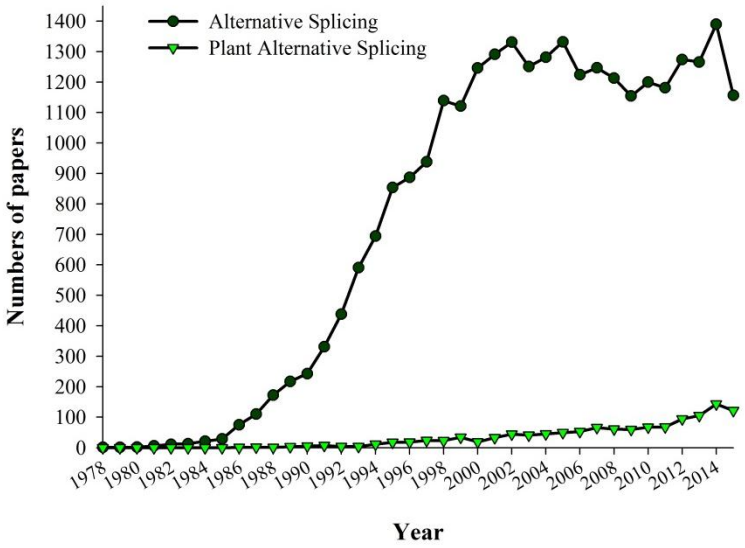

Figure 1. **Publications about alternative splicing and its evolution between 1978 and 2015.** Number of abstracts in PubMed with the word combination "alternative splicing" and "plant alternative splicing".

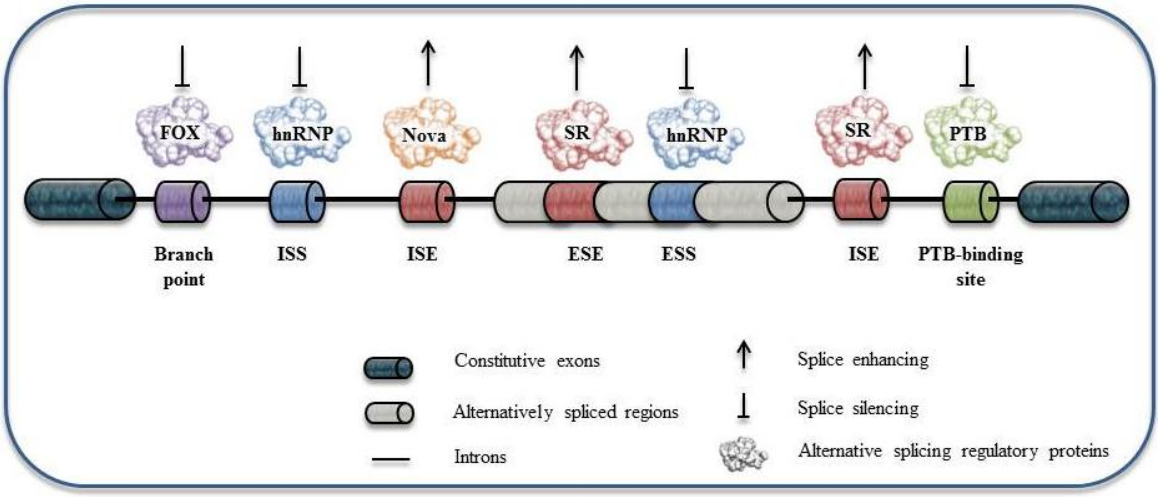

Figure 2. Alternative splicing regulation. Part of the alternative splicing regulation is promoted by *cis*-regulatory sequences called intronic silencing silencers (ISSs), intronic splicing enhancers (ISEs), exonic splicing enhancers (ESEs) and exonic splicing silencers (ESSs), besides some specific sites as branch point and PTB-binding site. These sites is binding by alternative splicing regulatory proteins as Ser/Arg-rich proteins (SRs), heterogeneous nuclear ribonucleoproteins (hnRNPs), polypyrimidine tract-binding (PTB) proteins, FOX proteins and NOVA proteins. Adapted from Kornblihtt et al. (2013) and Keren et al. (2010).

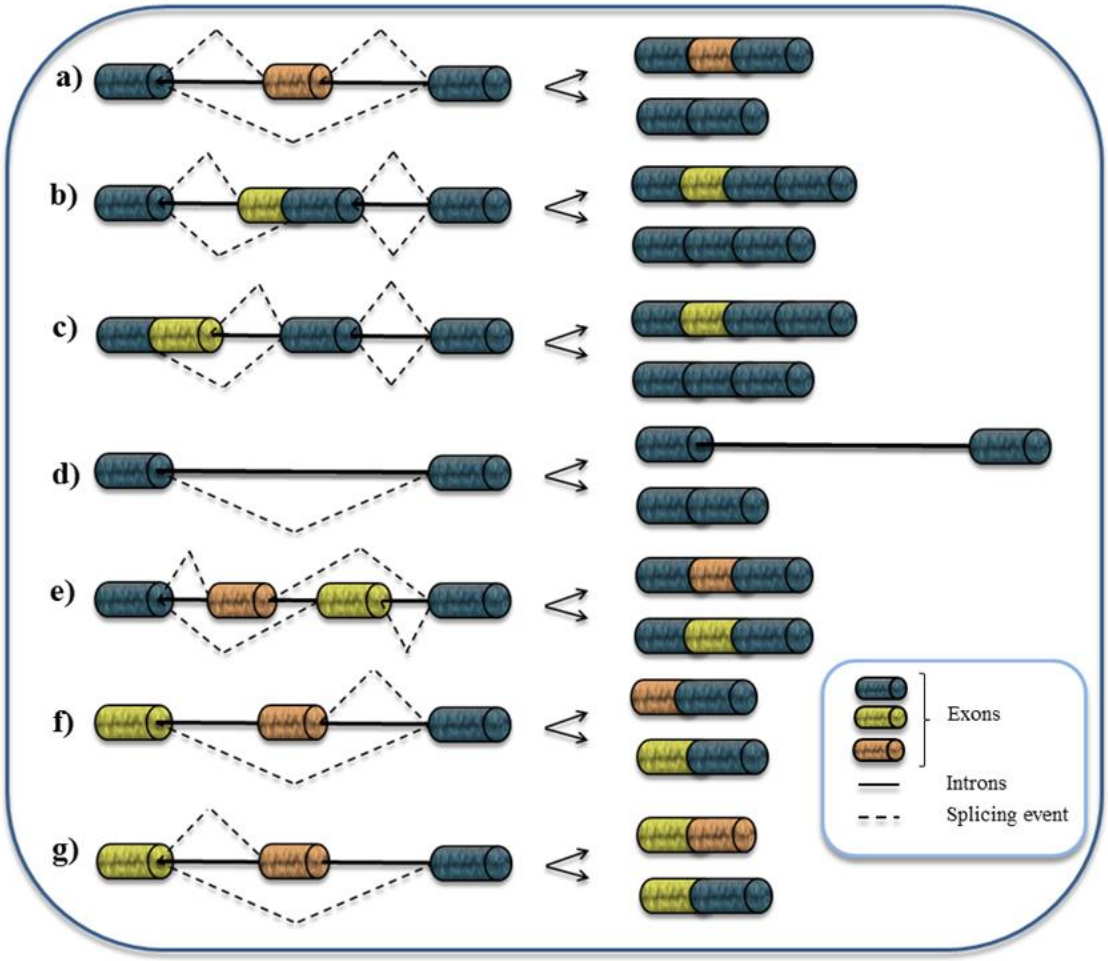

Figure 3. Types of Alternative splicing (AS) events. a) Exon skipping. B) Alternative 3′splice site (3′SS) selection. c) Alternative 5′splice site (5′SS) selection. d) Intron retention. e) Mutually exclusive exons. f) Alternative promoter. g) Alternative polyadenylation. Adapted from Keren et al. (2010).

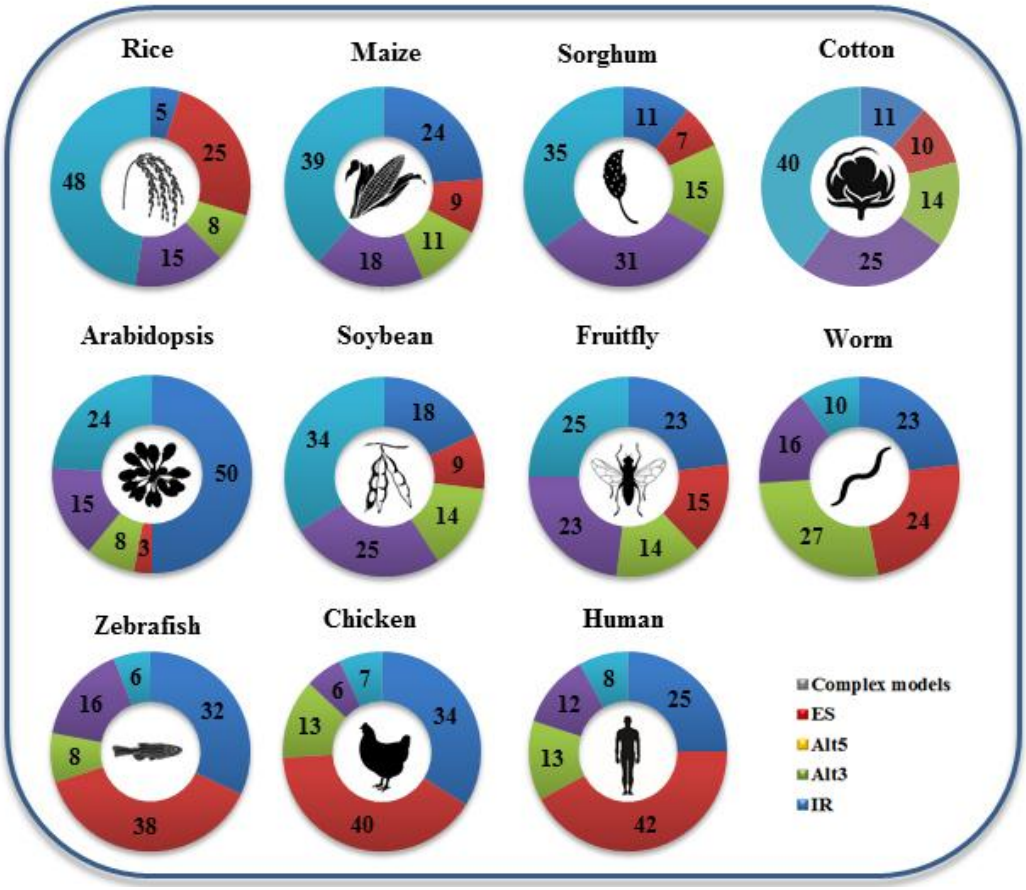

Figure 4. Proportion of alternative splicing types in different organisms. The four major AS types (Exon skipping (ES), alternative 5′splice site selection (Alt5), alternative 3′splice site selection (Alt3) and intron retention (IR)) and the complex AS models. Adapted from Li et al. (2014).

4.4 Artigo 4 – The quest for more tolerant rice: how high concentrations of iron affect alternative splicing?

Artigo publicado à revista *Transcriptomics: Open Access*

Artur Teixeira de Araujo Junior[1]; Daniel da Rosa Farias[1]; Railson Schreinert dos Santos[1]; Marcelo Nogueira do Amaral[3]; Luis Willian Pacheco Arge[1]; Danyela de Cássia Oliveira[1]; Solange Ferreira da Silveira Silveira[1]; Rogério Oliveira de Sousa[2]; Eugenia Jacira Bolacel Braga[3]; Luciano Carlos da Maia[1]; Antonio Costa de Oliveira[1]

[1]Plant Genomics and Breeding Center, Universidade Federal de Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil. Phone: 0555332757263, Fax: 0555332759031, acostol@terra.com.br

[2] Department of Soils, Universidade Federal de Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil

[3] Department of Botany, Universidade Federal de Pelotas, Campus Capão do Leão, 96010-900 Capão do Leão, Brazil

Abstract

Rice (*Oryza sativa* L.) is a global staple food crop and an important model organism for plant studies. Recent researches have shown that alternative splicing of different genes is affected by many stressful conditions, suggesting that this phenomenon can be an important way to adapt to adverse environments. Due to the little information on this subject, this study aimed to explore changes in splicing patterns that occur in response high concentration of iron in nutrient solution. Here we quantified different kind of junctions and splicing events in the transcriptome of a relatively tolerant rice cultivar BRS Querência, under iron excess with concentration of 300 mg L^{-1} Fe^{+2}. Plants kept under standard conditions (control) presented 127,781 different splicing junctions, while stressed plants had 123,682 different junctions. Canonical junctions accounted for 98.85% of the sites found in control plants while 98.91% of stressed, semi-canonical (0.73% for control and 0.70% for stressed) and non-canonical junctions (0.42% for control and 0.40% for stressed). Intron retention was the most frequent event in both situations (with 10,281 or 44.1% for control and 3,909 or 47.4% for stressed

plants), followed by 3' splice site (5,261 or 22.6% and 1,802 or 21.9%), exon skipping (4,413 or 18.9% and 1,429 or 17.3%) and alternative 5' splice site (3,352 or 14.4% and 1,104 or 13.4%). We also found 25 differentially expressed genes (five up and 20 down-regulated) that are related to post-translational modifications. These results represent an important step in the understanding of how plant stress responses occur in an iron tolerant genotype, with information that has not yet been published.

Keywords: transcriptomics; iron toxicity; abiotic stress

Introduction

Rice (*Oryza sativa* L.) is the second most widely grown cereal and one of the most important food sources for human nutrition. Most of rice production is concentrated in Asia. However, Brazil is the major producer outside this continent [1,2]. It is also important to notice that, apart from its social and economic importance, rice has been widely used as a model organism to investigate many aspects of plant biology, thus, much of the knowledge acquired from this species can also be applied to other monocots.

Several minerals are essential for plant growth and most of these are usually uptaken by roots, directly from the soil solution. Among these minerals, iron (Fe, from the Latin word "*ferrum*"), is a vital element for plant and animal life [3,4]. However, iron can be harmful when present at high concentrations [5], reason why its content is subjected to a precise control by the cells [6,7]. Iron excess is one of the most important constraints to rice production on soils irrigated by flooding. The low oxygen environment favors the reduction of this mineral, resulting in ferrous iron (Fe^{+2}) [5], a quite soluble form present in higher concentrations in the soil solution. This excessive iron can be easily absorbed and transported along the xylem, carried by the transpiration stream, leading to the generation of reactive oxygen species (ROS) in leaves, where the first symptoms are observed. Different physiological disorders caused by this toxicity culminate with big losses in crop production [8,9].

To survive unfavorable conditions, plants actively employ pre-mRNA splicing as a mechanism to regulate expression of stress-responsive genes, reprogramming intracellular regulatory networks [10]. RNA splicing is a biological process responsible for removing

introns from pre-mRNA, and joining exons. Splicing can lead to the generation of multiple protein isoforms from a single gene due to alternate use of 5' and/or 3' splice sites by the spliceosome, a phenomenon called alternative splicing (AS) [11,12]. In this sense, it is important to highlight the role of the splicing sites, the nucleotide sequences surrounding exon-intron boundaries that determine the action of the spliceosome [13].

AS is an important post-transcriptional regulatory mechanism that modulates gene expression and, eventually, protein forms and functions [8,9]. This process consists in a post-transcriptional modification responsible for increasing the diversity of proteins generating two or more mRNA variants of a single gene causing the inclusion or exclusion of peptide sequences, and modulation of gene expression by producing mRNA variants [14].

Plant AS events have not been characterized in many different conditions, but there is strong evidence indicating that these can promote fast changes that can contribute to species-specific differences and adaptation, performing important roles in many events, including stress response [15-18]. Recent studies suggest that environmental stresses can induce AS or alter its efficiency and fidelity in a number of genes playing a role in plant stress response and tolerance [19-22].

The progress achieved in rice transcriptome analysis has enabled researchers to understand expression patterns and their relation to function and regulation, as well as the relationship between global transcription and chromosome features [23]. Recent studies pointed out the extensive diffusion of these phenomena in plants and its importance in gene expression and stress response [24]. RNAseq technique has been used to achieve different objectives, including gene prediction [25,26], isoform identification and quantification [19,27-30], as well as discovery of non-coding transcripts [31-33].

Therefore, understanding transcriptome dynamics is essential for unveiling functional elements of the genome and interpreting phenotypic variation produced by combinations of genotypic and environmental factors [28]. Due to the little information available on this subject, in this study, we explore changes in splicing patterns that occur in response to iron excess, aiming to evaluate and quantify different kinds of junctions and alternative splicing events in the transcriptome of an iron-tolerant rice indica cultivar (BRS Querência) under high concentrations of iron.

Materials and Methods

Plant material

Seeds of rice (cv. BRS Querência) were germinated in a growth chamber for 7 days with a photoperiod of 16 hours and a temperature of 25 ± 2°C. After this period, seedlings were transferred to plastic trays (3 L) containing pre-washed sand, and kept in a greenhouse where irrigation, with water and nutrient solution [34], was maintained. Plants were subjected to iron treatment at the seedling stage [35], adding 300 mg L^{-1} Fe^{+2} to the nutrient solution [36], while untreated plants remained in normal nutrient solution (control). Plant material was collected 24 hours after iron treatment, frozen in liquid nitrogen and stored at -80°C for later RNA extraction.

Preparation of cDNA and sequencing

The total RNA was extracted from 100 mg of leaves, using Purelink® Plant RNA Reagent. TruSeq RNA Sample Preparation v2 (Illumina®) kit was used for the preparation of libraries, following manufacturer's recommendations. RNA-Seq analysis was performed using Illumina HiSeq 2500 platform (Illumina®) with paired-end 2 x 100 reads.

Data analysis

In order to evaluate read quality, the software FastQC Ver. 0.11.2 [37] was used. The Trimmomatic Ver. 0.32 [38] software was used to remove the adapters and low quality base reads of each library. Then, reads were mapped against the reference genome of *Oryza sativa* cv. Nipponbare (IRGSP build 1.0) from The Rice Annotation Project Database (RAP-DB) [39], by software TopHat Ver. 2.0.11 [40] with the argument "--report-secondary-alignments" to output additional or secondary alignments. TopHat uses software Bowtie Ver. 0.12.7 [41] to map the reads. To discover splice junction we mapped reads in the reference with MapSplice program [42] with default arguments, this software detected novel canonical, semi-canonical and non-canonical splice junctions. After, The Python package SpliceGrapher [43] was used to predict alternative splicing patterns by loci, for this analysis first we reduced gene models and filtered alignments to reduce the false-positive (with sam_filter.py), after we

predicted splice graphs for all transcripts (with predict_graphs.py). Finally we compared the predicted with complete gene models to see how closely the predicted graphs match the complete gene models (with gene_model_to_splicegraph.py). Finishing the analysis, MapMan Ver. 3.5.1.R2 [45] and the Predicted Rice Interactome Network - PRIN [46] were used to detect changes in metabolism, and in protein-protein interactions respectively. The identification of differentially expressed genes (DEGs) was performed using edge R Ver. 3.8.5 [46,47,48]. The expression levels were normalized by reads per kilobase per million reads (RPKM) method, with p-value < 0.01.

Results and Discussion

Here 127,781 junctions sites were found in plants under normal conditions (control), and 123,682 were found in plants under stressful conditions. Significant difference in the proportion of these junction sites are found when these are classified into the three main described categories (Figure 1A). As expected the most sites are classified as canonical (98.85% for control plants and 98.91% for stressed), followed by the semi-canonical (0.73% for control and 0.70% for stressed) and non-canonical junctions sites (0.42% in control and 0.40% in stressed).

The lower number of junction sites found in iron stressed rice can be explained mainly by the high tolerance of the genotype used in this study. The AS is predominant in some gene families, while absent in others [49], in this sense we can have a larger number of genes expressed in plants under stressful conditions that must belong to families that did not present AS.

The analysis of 5’ and 3’ splice sites in all introns of *Arabidopsis* and rice indicates that these are very similar to human, with just some subtle differences in the frequencies of specific nucleotides at specific positions. In *Arabidopsis*, non-canonical splicing sites occur in only 0.7% of all splicing sites [13]. Other reports demonstrated that 97.5% are canonical GT-AG pairs, while 1% is GC-AG and 1.5% a combination of less frequent non-canonical sites [24].

Non-canonical AS comprised “variations affecting multiple exons”, which refer to deletions of large coding sequences spanning several exons, intraexonic deletions and generation of chimeric mRNAs. These join together portions of two separate mRNA

molecules frameshifting, which was caused by an intron excision, therefore a lower number of non-canonical sites junctions can be a defense strategy against the stress in this cultivar [10].

The four major kinds of alternative splicing (intron retention, exon skipping, alternative 5' splicing site, and alternative 3' splicing site), that cover the vast majority of the alternative splicing events [50], were investigated in control and Fe overload plants. A deeper alternative splicing analysis showed that the control plants presented a higher number of events (23,307) when compared to the treated ones (8,244). The AS events were organ-specific, indicating a strong association of AS events with organ- specific regulation and a major role for AS in the functional complexity of plants [28], being this one of the possible reasons that for having a low number of events in the treated samples. Furthermore, it is interesting to note that intron retention was the most frequent event in both situations, with 10,281 (44.1%) for control and 3,909 (47.4%) for stressed (Figure 1B). Similar results were also detected in others studies, demonstrating that intron retention is the most frequent type of AS in plants [34,36,51]. The second major event is alternative 3' splice site (5,261/22.6% and 1,802/21.9%), followed by exon skipping (4,413/18.9% and 1,429/17.3%) and alternative 5' splice site (3,352/14.4% and 1,104/13.4%).

The increasing of the percentage of intron retention may be a reflect of the underlying mechanism to create novel function acting on many other genes in plants. The stress condition can affect the plant mechanisms, even with low effect, regulating splicing by multiple mechanisms, including alteration of the population or distribution of splicing factors, or induction of changes in phosphorylation status or expression of serine-arginine-rich protein [49].

The intron retention is the AS events observed with a presence of ~40% in *Arabidopsis* and rice, while only 9% is observed in human [52]. The most abundant splicing event in human is exon-skipping (42%) whereas in plants this event considered relatively rare, as also observed in experiment data in our study. This suggests that the splice regulatory components can influence the splicing mechanisms making difference between plant and animals. This data can be related to the hypothesis that organisms that typically have small introns use an intron-definition splicing mechanism, performing most of the AS events the intron retention, in contrast the organisms that have large introns use an exon definition mechanism, being thus the most used the exon skipping [53].

Similar results were already found in which intron retention represents 47% of all splicing events in rice [28]. Other reports also found that the most common event is intron retention, involving 77% of the alternatively spliced genes in grape [24]. The results obtained are consistent with other studies [19,27,28,51] supporting the idea that intron retention is a common event in plants.

The intron retention events and alternative 5' and 3' splice sites can produce more mRNAs with premature termination codons (PTCs). The presence of PTCs may result in mRNA degradation via the nonsense-mediated mRNA decay (NMD), and translation of truncated proteins [22,54,55]. Truncated proteins can have important roles in plant stress adaptation, once that truncated proteins derived from the PTC containing mRNAs are not necessarily functionless compared to the full-length protein [37,56,57].

The AS also affects a range of regulatory genes that are presumed to have important roles in abiotic stress adaptation [10]. Its influences are present in almost all aspects of protein functions, making it a central part of gene expression regulation. Recent studies suggest that epigenetic regulation not only determines which parts of the genome are expressed, but also how they are spliced. ROS and reactive nitrogen species (RNS) at an increased rate during stress, participating in signal transduction, modify cellular components and cause damage, being an important group of genes to analyze [58]. In this case, changes promoted by AS in the expression of proteins related to post-translational modifications were analyzed. These proteins are responsible for diversifying the functions of others proteins and for the dynamic coordination of signaling networks [40].

A total of 25 differentially expressed genes were found to be related to post-translational modifications (Table 1), of these, the first five genes (*Os01g0631700*, *Os04g0660500*, *Os02g0777800*, *Os07g0693000*, *Os03g0125600*) are up-regulated, while the remaining 20 are down-regulated. When these proteins were analyzed, kinases and phosphatases were found to be the most common classes, where 14 assignments were found. These proteins are responsible for two important biological processes: phosphorylation with 14.29% of the assignments (with a total of three down-regulated genes) and dephosphorylation with 85.71% of the assignments (where 14 genes were down-regulated and four were up-regulated). The most common molecular functions were transferase activity and ATP binding. In both cases, 16 down-regulated and four up-regulated assignments were observed. A change of scenario in genes responsible for post-translational modifications can be seen when plants are subjected to stress. This change is perceived by decreasing the

expression of most of these genes and by the activation of some other post- translational genes. These changes can be a differentiated response of plants to stress.

The activated post-translational modification genes are distributed into two main groups: protein kinases (*Os01g0631700*, *Os04g0660500* and *Os03g0125600*) and receptor-like cytoplasmic kinases (*Os02g0777800* and *Os07g0693000*). Protein kinases play key roles in most cellular activities, performing the phosphorylation in protein side chains, a process that usually results in a functional change of the target protein by changing enzyme activity, cellular location, or association with other proteins [59,60]. Receptor-like cytoplasmic kinases (RLCKs) have important roles in plant development and stress responses, where differential expression patterns suggest their involvement in diverse functions in rice [61]. These reports aid the understanding and confirmation of results obtained.

Plants respond to adverse conditions through a series of signaling processes that often involves diverse protein kinases. We found calcineurin B-like protein-interacting protein kinases (CIPKs), mitogen-activated protein kinases (MAPKs), stress-activated protein kinase and receptor-like cytoplasmic kinases were down-regulated. Differential expression of these genes had already been detected for stresses like drought, salinity, cold, polyethylene glycol, or abscisic acid treatment [62-65].

Just one locus (LOC_Os02g34600) showed interactions with other differentially expressed genes. Correlations with other six loci (Figure 3A), five negative (co-expression values between -0.1879 and -0.0210), and one positive correlation (0.1756) were found. Metabolomic correlation networks, indicated negative co-expression between folding protein and post-translational modification proteins (Figure 3B). Post-translational modifications had positive co-expression with proteins of RNA processing also had negative co-expression with ribosomal protein synthesis and some proteins with metabolism non-assigned.

Conclusion

Rice plants of cv. BRS Querência under stress with concentration 300 mg L^{-1} Fe^{+2} during 24 hours change the pattern of alternative splicing in leaves. Also, changes in the expression of post-translational modification genes occur. Changes of AS events may indicate a new tolerance strategy of plants. This work represents an important step for understand how the rice plant respond to stress and how this response can increase tolerance in this species.

More studies are necessary in order to elucidate the influence of these up-regulated genes on plant tolerance responses under stress by iron overload. Alternative transcripts identified here constitute important targets for molecular breeding through genetic engineering and marker development.

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Competing interests

The authors have no potential competing interests to declare.

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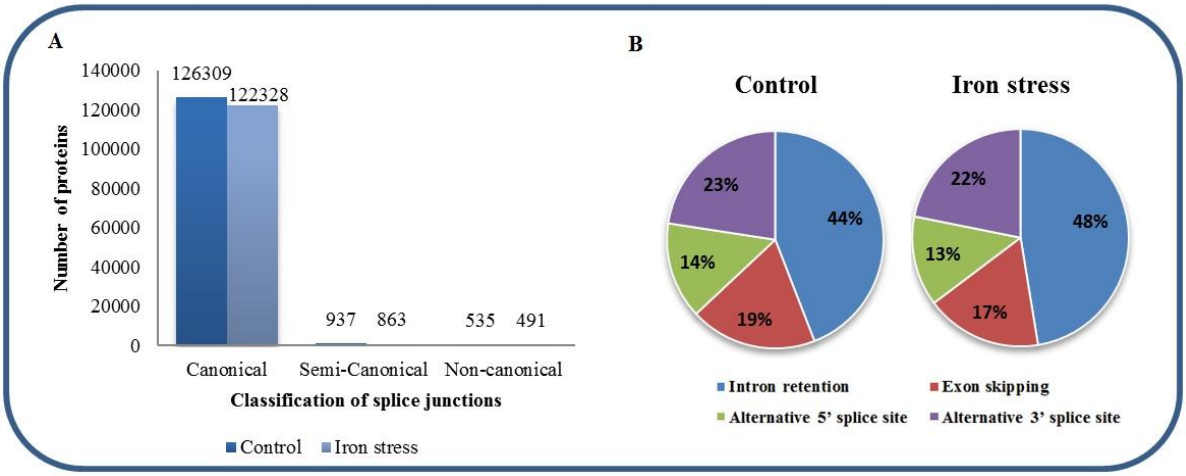

Figure 1 Alternative splicing analysis in leaves of rice seedling stage (*Oryza sativa* ssp. *indica* cv. BRS Querência) after 24 hours of iron excess exposure. (A) Types of alternative splicing junctions sites. (B) Pie chart showing the percentage distribution of alternative splicing events in control a stress condition (300 mg L^{-1} Fe^{+2}).

Table 01 Genes related to post-translational modifications which are differentially expressed in rice (*Oryza sativa* ssp. *indica* cv. BRS Querência) under iron excess (300 mg L^{-1} Fe^{+2}).

Gene-ID	Locus	Description
Os01g0631700	LOC_Os01g44110	Similar to Ser Thr specific protein kinase-like protein
Os04g0660500	LOC_Os04g56530	Armadillo-type fold domain containing protein
Os02g0777800	LOC_Os02g53750	Protein kinase, catalytic domain domain containing protein
Os07g0693000	LOC_Os07g49240	Similar to Ser-thr protein kinase
Os03g0125600	LOC_Os03g03410	Ser Thr specific protein kinase-like protein
Os04g0487200	LOC_Os04g41030	Serine/threonine protein kinase domain containing protein
Os07g0150700	LOC_Os07g05620	Serine/threonine protein kinase
Os12g0203000	LOC_Os12g10190	Similar to Cyclin-dependent protein kinase-like protein
Os11g0207200	LOC_Os11g10100	Similar to MAP3Ka
Os05g0318700	LOC_Os05g25450	Similar to Resistance protein candidate
Os04g0691100	LOC_Os04g59450	Serine/threonine-protein kinase SAPK5
Os04g0490500	LOC_Os04g41310	Protein kinase-like domain domain containing protein
Os02g0551100	LOC_Os02g34600	Serine/threonine-protein kinase SAPK6
Os07g0283125	LOC_Os07g18240	Similar to lectin-like receptor kinase 7
Os01g0155500	LOC_Os01g06280	Protein kinase, catalytic domain domain containing protein
Os02g0787300	LOC_Os02g54600	Similar to MAP kinase kinase
Os01g0759400	LOC_Os01g55450	CBL-interacting protein kinase 12
Os01g0960400	LOC_Os01g72990	Protein kinase, core domain containing protein
Os03g0634400	LOC_Os03g43440	Serine/threonine protein kinase domain containing protein
Os01g0583100	LOC_Os01g40094	Similar to Protein phosphatase 2C
Os01g0818700	LOC_Os01g60280	Leucine-rich repeat, N-terminal domain containing protein
Os03g0772600	LOC_Os03g56160	Similar to Lectin-like receptor kinase 7;2
Os01g0655500	LOC_Os01g46720	Protein kinase, core domain containing protein
Os02g0799000	LOC_Os02g55560	Similar to DNA-binding protein phosphatase 2C
Os03g0339900	LOC_Os03g22050	Similar to Serine/threonine protein kinase

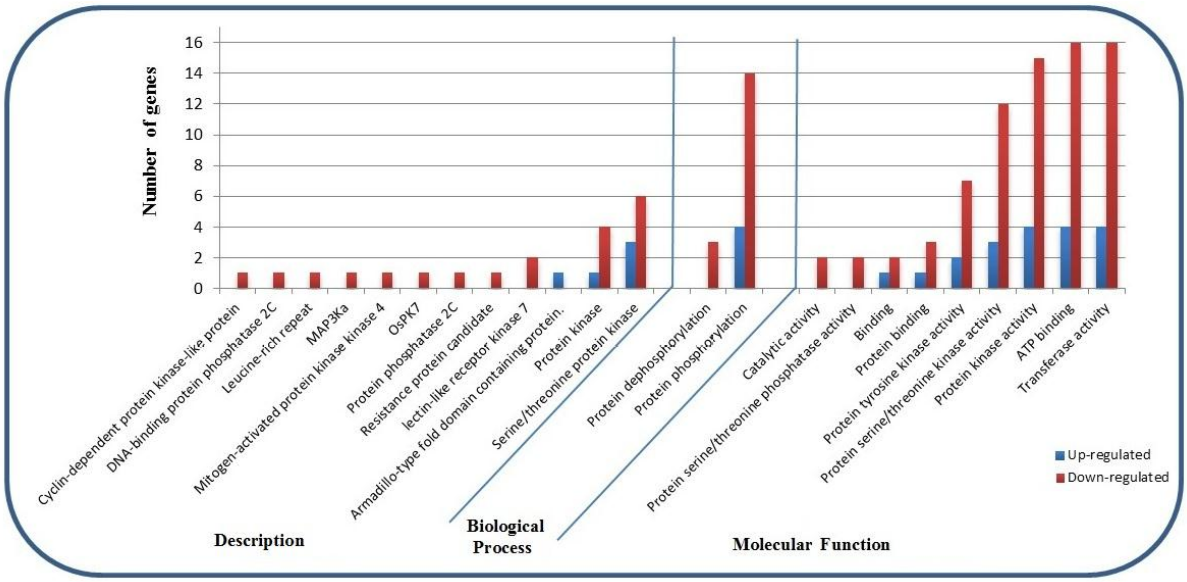

Figure 2 Gene ontology of differentially expressed genes related to post-translational modifications in rice (*Oryza sativa* ssp. *indica* cv. BRS Querência) under iron excess (300 mg L^{-1} Fe^{+2}).

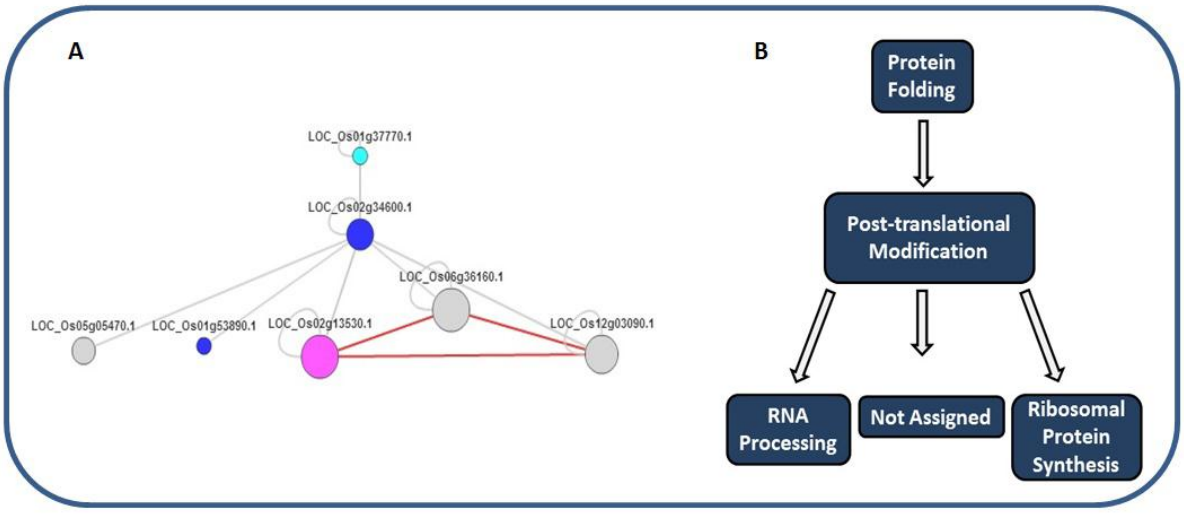

Figure 3 Interactions between genes involved in post-translational modifications in rice leaves at seedling stage (*Oryza sativa* ssp. *indica* cv. BRS Querência) after 24 hours of exposure to high concentrations of iron (300 mg L^{-1} Fe^{+2}). (A) Protein-protein interaction networks. Gray lines represent co-expression less or equal to 0.5 and red lines represent co-expression above 0.8. The circle of colors representing their possible subcellular localization: navy blue to mitochondria, dark blue for the nucleus, pink for cytoplasm and gray for unknown localization. (B) Co-expression metabolomics network.

5 DISCUSSÃO GERAL E PERSPECTIVAS

O alagamento do solo pode promover a solubilização do ferro, desencadeando em um acúmulo de Fe(II) na solução do solo, podendo atingir níveis tóxicos (Becker e Asch, 2005). A toxidez por ferro pode ocorrer na planta de duas formas distintas: por absorção excessiva (toxidez direta ou bronzeamento) ou por deficiência nutricional múltipla (toxidez indireta ou alaranjamento). Por mais que se tenha estudos com a finalidade de identificar a resposta da planta frente a este estresse, analisando tanto os efeitos morfológicos quanto as alterações no transcriptoma, ainda existe uma carência de informação em relação ao mecanismo de homeostase de ferro. A resposta da planta depende da cultivar analisada, das condições de estresse, do estádio de desenvolvimento e do tipo de tecido. Sendo de grande relevância entender como o excesso deste metal afeta o metabolismo e o transcriptoma das folhas.

A cv. de arroz BRS Querência foi caracterizada como tendo bons níveis de tolerância ao excesso de Fe e sob as condições adotadas neste trabalho foi possível verificar alterações no perfil transcricional das folhas, ocorrendo tanto a ativação quanto a repressão de uma grande quantidade de genes, observando-se assim, mudanças em um grande número de vias metabólicas. Ao longo desse trabalho foi realizada uma interpretação dos dados e de como essas alterações desencadearam uma possível resposta de tolerância.

Dos genes expressos diferencialmente, obteve-se uma alta ativação dos que codificam proteínas relacionadas à homeostase de ferro, responsáveis principalmente, pelo transporte, quelação e armazenamento. Dentre os vários mecanismos de tolerância que se conhece atualmente, tem-se que a ativação deste conjunto de genes pode estar relacionada ao mecanismo do Tipo II, no qual, o Fe(II) é absorvido e armazenado numa forma menos reativa. A BRS Querência elevou em aproximadamente 12 vezes a expressão do gene que codifica a proteína VACUOLAR IRON TRANSPORTER 1 (VIT1), um transportador de membrana envolvido na captação e translocação de Fe para o vacúolo, sendo que esta elevação também foi encontrada em outros trabalhos sobre excesso de ferro (Quinet et al., 2012; Finatto et al., 2015). O gene que codifica a proteína FERRITIN teve sua expressão elevada em torno de quatro vezes. Sua função está relacionada ao armazenamento de Fe, podendo tanto fornecer Fe para síntese de novas proteínas

quanto armazenar o Fe numa forma menos reativa, evitando possíveis danos causados por radicais livres (Goto et al., 1999).

Além disso, foi possível observar a ativação tanto do gene codificador à proteína FERRIC REDUCTASE DEFECTIVE 3 (FRD3), que é responsável por quelar o Fe e disponibilizá-lo para a parte aérea em uma forma utilizável, quanto do gene que codifica a METHYLTHIOADENOSINE NUCLEOSIDASE (MTN), uma enzima reguladora do ciclo da metionina, no qual, está associado com a biossíntese de etileno, um importante hormônio que atua na resposta ao estresse (Bürstenbinder et al., 2010; Wang et al., 2013). Todavia, é necessário mais análises, principalmente uma confirmação em laboratório, para assim, permitir a compreensão detalhada da ação da expressão desses genes nas folhas, verificando se não ocorre também um aumento da expressão de outros genes relacionados à homeostase de Fe. Vale ressaltar que diferentes mecanismos de tolerância podem ocorrer na planta, dependendo do tecido analisado e do estádio de desenvolvimento. Desta forma, seria de grande valia analisar os perfis de expressão de diferentes tecidos submetidos à condição de estresse em diferentes estádios de desenvolvimento, para assim, ter-se um entendimento mais completo de como é desempenhada a resposta de tolerância nesta cultivar.

A resposta da planta ao estresse envolve vários rearranjos de processos metabólicos e fisiológicos que devem adaptar o organismo vegetal ao ambiente alterado. Os resultados obtidos neste experimento demonstram uma grande quantidade de metabolismos que foram afetados, reforçando a ideia de uma complexa rede de resposta, ocasionando uma expressão diferenciada de diversos genes. Entre essas alterações, temos a importante classe de genes diferencialmente expressos que codificam as HEAT SHOCK PROTEINS (HSPs), nas quais, encontraram-se nove genes diferencialmente expressos que codificam essas proteínas, sendo cinco ativados e quatro reprimidos. Além disso, foi analisado os genes responsáveis pelas proteínas sinalizadoras, observando-se uma repressão dos que codificam as receptoras de quinase. Estas duas classes de genes fazem parte do grupo de proteínas reguladoras responsáveis por desencadear um rearranjo nos diferentes metabolismos da célula, auxiliando na resposta ao estresse. As HSPs desempenham um importante papel na resposta da planta aos estresses abióticos, possuindo interações com diversos processos celulares, auxiliando na proteção da planta (Iba, 2002; Pegoraro et al., 2011). As proteínas sinalizadoras possuem a

função de perceber estímulos extracelulares e transmitir sinais de resposta a estes estímulos, desencadeando a expressão ou repressão de diferentes genes (Shiu & Bleecker, 2001; Morris & Walker, 2003). O perfil transcricional obtido no experimento sugere uma resposta que pode ser única da cultivar analisada, onde se tem a repressão dos genes associados às proteínas receptoras de quinase e a ativação de um conjunto de HSPs específico. Desta forma, mais estudos são necessários para se obter uma maior compreensão de quais metabolismos foram alterados devido a resposta destas duas classes de genes.

Outros grupos importantes de genes que foram afetados pelo estresse são os que codificam proteínas da fotossíntese e do metabolismo hormonal. A fotossíntese é descrita como um dos metabolismos mais sensíveis aos estresses abióticos. Isso ocorre devido à importância que este metabolismo tem para a planta e pela diversidade de proteínas que estão envolvidas neste processo. Dentre os diversos componentes, o Fotossistema II (PSII) é um dos mais sensíveis, tendo grandes danos proporcionados pela geração de espécies reativas de oxigénio (ROS), no qual, pode resultar em uma fotoinibição (Wargent et al., 2013; Gururani et al., 2015). Assim, analisar as alterações deste mecanismo pode auxiliar na compreensão de como é realizada esta resposta para preservar as funções das proteínas envolvidas. Os resultados obtidos demonstram que na cultivar BRS Querência ocorre uma ativação dos genes relacionados às subunidades do PSII e a repressão dos genes que codificam o LIGHT-HARVESTING COMPLEX II, indicando um possível reparo na maquinaria do PSII, protegendo contra os danos promovidos pelas ROS. Contudo, para reforçar essas afirmações é necessário realizar-se mais análises em laboratório verificando essas alterações e suas consequências para a sobrevivência da planta.

Ademais, tem-se a grande relevância do metabolismo hormonal na resposta ao estresse, sendo responsáveis por prováveis alterações no perfil de expressão gênica e nos processos celulares. No presente estudo, obteve-se mudanças na ativação dos genes relacionados ao etileno e a repressão dos genes relacionados ao ácido abscísico e às auxinas. Parte dessa resposta pode ter ocorrido devido à expressão similar dos fatores de transcrição desses genes. O aumento da síntese de etileno pode estar relacionado a uma resposta adaptativa, ajudando na sobrevivência da planta em condições adversas, tendo a influência dos fatores de resposta de etileno, os quais, são elementos de resposta primária e atuam também

como reguladores de outros genes (Dugardeyn et al., 2008; Santos et al., 2013). Com isso, mais estudos deverão auxiliar no entendimento de como estas alterações hormonais podem promover uma resposta de sobrevivência da planta e quais as classes de genes são reguladas por estas modificações na expressão.

Considerando a importância de estudos de expressão gênica com suas associações as diferentes respostas fisiológicas da planta, é de grande relevância ter avanços na área de bioinformática, para auxiliar no manuseo dos dados e no entendimento das alterações dos diversos metabolismos. Dentre os avanços obtidos, tem-se o desenvolvimento da tecnologia do RNA-Seq, o qual, além de fornecer dados da expressão diferencial dos genes também é capaz de auxiliar nas análises de *splicing* alternativo que estes genes podem realizar.

Diversos estudos indicam que alterações no ambiente podem desencadear mudanças no padrão de *splicing*, sendo que as consequências dessas alterações podem proporcionar uma regulação na expressão dos genes e na formação de proteínas que fazem parte da resposta de tolerância (Reddy, 2007). Os resultados desta análise demonstram que a BRS Querência teve uma diminuição tanto na quantidade do tipo de junção do *splicing* alternativo quanto nos principais tipos de eventos de *splicing*. Essas mudanças podem estar diretamente relacionadas como sendo uma resposta adaptativa típica desta cultivar, do tecido e/ou do estádio de desenvolvimento analisado, promovendo mudanças específicas e pontuais, não tendo assim, perdas de energia na produção de novos variantes de *splicing*, no qual, não possuem função direta com a resposta ao estresse. Contudo, maiores estudos voltados para análises de bioinformática deverão ser realizados para determinar quais proteínas ou RNAs contribuíram para esta diminuição, podendo assim, determinar-se se houve a ação de algum fator de regulação, proporcionando estudos mais detalhados no laboratório.

Compreender as respostas das plantas aos estresses abióticos é um tema importante e desafiador. Com base nos diversos progressos alcançados através de pesquisas, obteve-se como consequência primordial as melhorias na tolerância das plantas aos estresses, tendo a identificação de importantes genes e sua utilização nas transferências genéticas e seleção assistida por marcadores.

Os resultados decorrentes deste experimento representam um passo importante para a compreensão dos mecanismos de tolerância da planta ao excesso de ferro, despertando um interesse para maiores estudos das consequências que

esses genes diferencialmente expressos aqui mencionados podem promover na célula e sua relação com o processo adaptativo da planta, podendo futuramente, determinar possíveis genes alvos para uma caracterização no laboratório e sua utilização nos programas de melhoramento.

6 CONCLUSÃO GERAL

Os resultados obtidos neste estudo evidenciam que a tolerância ao estresse por ferro na cultivar de arroz BRS Querência parece estar associada à expressão diferenciada de um grande número de genes, dentre esses o aumento na expressão dos genes que codificam proteínas responsáveis pelo transporte, quelação e armazenamento de ferro, genes que codificam proteínas de choque térmico e do fotossistema II. Obteve-se também a repressão de genes que codificam proteínas associadas ao metabolismo hormonal, relacionados ao ácido abscísico e às auxinas, e de sinalização, tendo em destaque os que codificam proteínas receptoras de quinases. Além disso, ocorreram alterações no padrão de *splicing* alternativo, tendo uma redução tanto a quantidade de sítios de junção quanto de eventos de *splicing*. Este estudo proporcionou a identificação de diversos genes que estão envolvidos na resposta de toxicidade de ferro em arroz, e que apresentam um potencial para futuros estudos de transformação genética.

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8 ANEXOS

Anexo A – Additional file 1 (Artigo 2)

Additional file 1. List of genes differentially expressed in rice (cv. BRS Querência) under excess of Fe.

Locus-ID	Log_2FC	Description
Os08g0520000	-2.670	Protein brittle- chloroplastic amyloplastic-like
Os12g0569500	-1.749	Thaumatin family expressed
Os12g0580600	-1.625	Conserved hypothetical protein
Os01g0708350	-1.571	Non-protein coding transcript
Os01g0855200	-1.538	Peroxidase-like protein
Os03g0402800	-1.519	Tify domain containing protein
Os07g0412100	-1.489	Granule-bound starch synthase II
Os03g0667100	-1.472	Transposon pong sub-class
Os01g0719800	-1.451	Hypothetical protein
Os03g0701200	-1.441	Similar to sugar-starvation induced protein (fragment)
Os02g0306401	-1.441	Nicotianamine aminotransferase
Os04g0547900	-1.407	Mutt domain
Os01g0123900	-1.332	Bowman birk trypsin inhibitor
Os05g0508400	-1.329	Jacalin lectin family protein
Os01g0695700	-1.325	Mdr-like abc transporter
Os04g0585200	-1.281	Glutamate receptor 3
Os06g0591400	-1.266	Unnamed protein product [*Oryza sativa japonica* group]
Os05g0429900	-1.252	Similar to mybhv5 (fragment)
Os09g0106700	-1.217	Similar to myb proto-oncogene protein (c-myb)
Os09g0509700	-1.185	Zinc finger, b-box domain containing protein
Os01g0600900	-1.185	Chlorophyll a-b binding protein 2
Os03g0339900	-1.178	Similar to serine/threonine protein kinase
Os01g0389700	-1.176	Protein of unknown function duf679 family protein.
Os03g0806600	-1.168	Conserved hypothetical protein
Os04g0623300	-1.168	Probable polyamine oxidase 2-like
Os05g0537100	-1.167	Wrky7 - superfamily of TFs having wrky and zinc finger domains
Os02g0799000	-1.157	Similar to DNA-binding protein phosphatase 2c
Os12g0493900	-1.130	Armadillo-like helical domain containing protein
Os04g0670600	-1.128	Exostosin family domain-containing protein
Os06g0606700	-1.112	Tetratricopeptide-like helical domain containing protein
Os07g0421932	-1.108	Tetratricopeptide repeat-containing protein
Os10g0536400	-1.097	Similar to oxidoreductase, 2og-fe oxygenase family protein
Os07g0185900	-1.092	Conserved hypothetical protein
Os07g0142900	-1.089	Aldo/keto reductase family protein
Os08g0111200	-1.080	Beta-glucosidase, gba2 type domain containing protein
Os04g0136700	-1.059	Cystathionine beta-synthase, core domain containing protein

... continuation

Locus-ID	Log_2FC	Description
Os08g0434300	-1.035	Nad-malate dehydrogenase
Os01g0872000	-1.034	Peptide transporter
Os09g0271100	-1.026	Conserved hypothetical protein
Os06g0180666	-1.024	Hypothetical gene
Os02g0124866	-1.013	Hypothetical gene
Os01g0810300	-1.005	Cam61, calmodulin_61; similar to calmodulin-like protein
Os01g0822800	-0.996	Similar to ring-h2 finger protein atl3c
Os01g0655500	-0.990	Protein kinase, core domain containing protein
Os02g0674233	-0.986	Hypothetical conserved gene
Os08g0249900	-0.980	Similar to gibberellin 20 oxidase 2
Os01g0138900	-0.975	L-ala-d l-glu epimerase-like
Os03g0741050	-0.970	Non-protein coding transcript
Os03g0140200	-0.960	Cytochrome p450 family expressed
Os12g0225150	-0.952	Hypothetical protein
Os07g0615200	-0.951	Tify domain containing protein
Os11g0208900	-0.942	lrr receptor-like kinase
Os04g0531750	-0.938	Salutaridine reductase-like
Os03g0766500	-0.936	Similar to two-component response regulator arr1
Os05g0453500	-0.926	Guanylyl cyclase domain containing protein
Os03g0184100	-0.926	Conserved hypothetical protein
Os07g0572100	-0.914	Copper amine oxidase
Os10g0524600	-0.914	Subtilisin-like protease sdd1-like
Os07g0137000	-0.914	Myb transcription factor domain containing protein
Os03g0772600	-0.905	Receptor kinase lecrk
Os12g0601800	-0.896	Similar to bzip transcription factor family protein
Os09g0251800	-0.889	Calcium-dependent lipid-binding domain-containing protein
Os05g0231700	-0.889	Similar to Tonoplast intrinsic protein.; K09873 aquaporin TIP
Os01g0717000	-0.888	Similar to gmck1p
Os02g0277700	-0.878	Probable lrr receptor-like serine threonine-protein kinase at4g08850-like
Os04g0282400	-0.873	Similar to fpf1 protein-like (raa1)
Os04g0671100	-0.872	Adenylate kinase
Os10g0479500	-0.866	Cytokinin riboside 5 -monophosphate phosphoribohydrolase log3
Os07g0534700	-0.866	Serine/threonine protein kinase-related domain containing protein
Os05g0110000	-0.864	E3 ubiquitin-protein ligase ring1-like
Os08g0508500	-0.864	Similar to predicted protein
Os02g0164000	-0.863	Peptidase c48, sumo/sentrin/ubl1 domain containing protein
Os03g0766800	-0.863	Conserved hypothetical protein
Os07g0164900	-0.861	Aldehyde oxidase
Os10g0574700	-0.860	Protein of unknown function duf597 family protein
Os01g0818700	-0.856	Retrotransposon line subclass
Os01g0583100	-0.849	Similar to protein phosphatase 2c
Os07g0273900	-0.848	Disease resistance protein domain containing protein
Os01g0922700	-0.834	Conserved hypothetical protein
Os10g0389500	-0.833	Conserved hypothetical protein

... continuation

Locus-ID	Log_2FC	Description
Os09g0346500	-0.825	Chlorophyll a-b binding chloroplast precursor
Os05g0589200	-0.825	Sigma factor
Os05g0513100	-0.820	Similar to plcyc4 protein (fragment)
Os01g0665900	-0.815	Conserved hypothetical protein
Os03g0348200	-0.814	Retrotransposon unclassified
Os07g0290200	-0.813	Pvr3-like protein precursor
Os01g0305200	-0.801	Lg106-like family protein
Os03g0110900	-0.801	Stress responsive alpha-beta barrel domain protein
Os02g0286933	-0.798	Conserved hypothetical protein
Os08g0434632	-0.797	Glucomannan 4-beta-mannosyltransferase 9-like
Os07g0619600	-0.797	Homeobox domain containing protein
Os03g0634400	-0.795	Cbl-interacting serine threonine-protein kinase 7
Os07g0476900	-0.789	Thioredoxin-like protein cdsp32
Os01g0730500	-0.785	Similar to ferredoxin
Os03g0766600	-0.781	Conserved hypothetical protein
Os11g0104350	-0.780	Hypothetical conserved gene
Os01g0678100	-0.779	Protein of unknown function duf2921 domain containing protein
Os03g0707250	-0.779	Non-protein coding transcript
Os08g0249300	-0.776	Cell wall integrity protein scw1
Os01g0819466	-0.770	Non-protein coding transcript
Os05g0127500	-0.769	Similar to leucoanthocyanidin dioxygenase-like protein
Os12g0556200	-0.767	Calmodulin binding protein-like family protein
Os01g0711400	-0.761	Glycine dehydrogenase
Os03g0411800	-0.758	Zinc transporter
Os05g0406150	-0.756	Inosine-uridine preferring nucleoside hydrolase
Os01g0202500	-0.752	Probable salt tolerance-like protein at1g78600-like
Os12g0104300	-0.751	Abc-1 domain containing protein
Os02g0704000	-0.749	Neoxanthin cleavage enzyme-like protein
Os09g0532500	-0.745	Pentatricopeptide repeat-containing protein
Os05g0508300	-0.741	Cysteine proteinase
Os01g0102850	-0.739	Similar to nitrilase 2
Os12g0569700	-0.735	Similar to heat shock protein 70
Os09g0287000	-0.734	Similar to ethylene-responsive transcription factor 5
Os08g0356500	-0.733	Protein of unknown function duf247
Os04g0448900	-0.730	Zeaxanthin epoxidase
Os01g0736900	-0.730	Seed maturation protein
Os10g0422300	-0.724	Pentatricopeptide repeat-containing protein mitochondrial-like
Os03g0741100	-0.720	Helix-loop-helix dna-binding domain containing protein
Os10g0501500	-0.709	Protein of unknown function duf607 family protein
Os10g0510500	-0.708	Auxin-responsive family protein
Os01g0960400	-0.707	Probable lrr receptor-like serine threonine-protein kinase at1g06840-like
Os07g0622200	-0.706	2-dehydro-3-deoxyphosphoheptonate aldolase 3-deoxy-d-arabino-heptulosonate 7-phosphate synthetase
Os02g0550600	-0.698	Plant neutral invertase family protein

... continuation

Locus-ID	Log_2FC	Description
Os11g0634200	-0.696	Conserved hypothetical protein
Os02g0254550	-0.695	Zinc finger, bed-type predicted domain containing protein
Os08g0480200	-0.691	2og-Fe(II) oxygenase domain containing protein
Os12g0507500	-0.689	Similar to swib/mdm2 domain containing protein
Os07g0684100	-0.689	Thioredoxin-like 1-1
Os04g0475200	-0.689	G-type lectin s-receptor-like serine threonine-protein kinase rlk1-like
Os02g0513800	-0.688	Uncharacterized protein loc100844763
Os02g0661800	-0.686	Presqualene diphosphate phosphatase-like
Os09g0434500	-0.684	Ethylene response factor
Os05g0100366	-0.682	Non-protein coding transcript
Os08g0374000	-0.681	Bet v i allergen family protein
Os06g0542150	-0.674	RNA-binding protein
Os01g0818600	-0.671	Probable lrr receptor-like serine threonine-protein kinase at1g06840-like
Os01g0316600	-0.671	Similar to pre-mRNA-splicing factor sf2
Os10g0396666	-0.666	Hypothetical conserved gene
Os04g0492500	-0.664	Rwd domain containing protein
Os03g0145900	-0.661	Ring finger and chy zinc finger domain-containing protein 1
Os07g0684400	-0.661	Hypothetical protein
Os07g0569600	-0.658	Chaperonin-like rbcx domain containing protein
Os11g0300400	-0.658	Similar to catalytic/ hydrolase
Os01g0717400	-0.652	Tryptophan aminotransferase-related protein 4-like
Os01g0759400	-0.652	Cbl-interacting serine threonine-protein kinase 11
Os04g0623600	-0.652	Peroxisomal glycolate oxidase
Os11g0157600	-0.650	Similar to cct motif family protein
Os03g0732100	-0.648	Bel1-type homeodomain protein
Os06g0186400	-0.646	Serine carboxylase ii-2
Os09g0380000	-0.645	Acetyl-coenzyme a synthetase-like
Os02g0299300	-0.642	Hypothetical protein
Os07g0660100	-0.642	Similar to predicted protein
Os03g0197100	-0.641	Polyol transporter 5-like
Os02g0575700	-0.640	Loricrin-like protein
Os03g0805600	-0.637	Iron-sulfur cluster-binding protein
Os04g0105400	-0.636	Unc93-like protein
Os02g0167600	-0.633	Saur25 - auxin-responsive saur family member
Os12g0514000	-0.632	Sugar transporter family expressed
Os10g0492900	-0.630	Alpha-galactosidase 1
Os05g0183100	-0.630	Similar to wrky transcription factor 16 (fragment)
Os07g0595700	-0.627	Hypothetical protein
Os06g0125800	-0.624	E3 ligase
Os07g0180300	-0.623	Protein of unknown function duf594 family protein
Os01g0279400	-0.623	Major facilitator superfamily antiporter
Os01g0708900	-0.623	Graves disease carrier
Os04g0493000	-0.622	Zinc finger protein
Os09g0456200	-0.621	Similar to bzip transcription factor abi5

... continuation

Locus-ID	Log_2FC	Description
Os07g0628900	-0.615	Receptor-like protein kinase 4
Os03g0146300	-0.614	Similar to cyclin-dependent kinases regulatory subunit
Os08g0190200	-0.609	Rna binding protein 45
Os03g0586500	-0.606	Chloroplast post-illumination chlorophyll fluorescence increase protein
Os11g0195500	-0.606	Lipase family expressed
Os07g0182200	-0.605	Transcription factor une12
Os09g0549450	-0.602	Similar to nin-like protein 1
Os04g0442800	-0.598	Similar to phragmoplast-associated kinesin-related protein 1
Os07g0558300	-0.598	3 -bisphosphate nucleotidase
Os01g0871500	-0.598	Oligopeptide transporter
Os01g0871800	-0.596	Tgf-beta receptor, type I/II extracellular region family protein
Os01g0645900	-0.595	Sulfate transporter
Os03g0168200	-0.594	Similar to f16a14.21
Os04g0606200	-0.593	Similar to osigba0113i13.1 protein
Os03g0646800	-0.592	Similar to dna-directed RNA polymerase
Os01g0772700	-0.591	Armadillo beta-catenin-like repeat-containing protein
Os03g0616400	-0.588	Calcium-transporting atpase plasma membrane-type
Os03g0169600	-0.586	Dof zinc finger
Os01g0847700	-0.585	Similar to aldose reductase
Os10g0330000	-0.584	Conserved hypothetical protein
Os08g0295100	-0.582	Ubiquitin domain containing protein
Os10g0447900	-0.581	Transmembrane expressed
Os12g0580700	-0.581	Similar to ring-h2 finger protein atl2n
Os02g0787300	-0.581	Map kinase kinase
Os03g0411100	-0.574	Ccaat-box transcription factor complex expressed
Os02g0475400	-0.572	Uncharacterized sodium-dependent transporter yocs-like
Os11g0130200	-0.569	Protein of unknown function duf309 family protein
Os05g0548900	-0.569	Phosphoethanolamine n-methyltransferase 3
Os03g0388500	-0.568	Similar to anther ethylene-upregulated protein er1
Os06g0686700	-0.566	Non-protein coding transcript
Os11g0197400	-0.565	Magnesium transporter nipa2-like
Os01g0128800	-0.558	Plant synaptotagmin
Os05g0298200	-0.556	Ankyrin repeat containing protein
Os04g0685300	-0.556	Harpin inducing protein
Os08g0191150	-0.554	Aconitate hydratase
Os07g0628700	-0.553	Receptor-like protein kinase 4
Os07g0170200	-0.553	Hnh endonuclease
Os01g0558850	-0.551	Chloroplast processing enzyme-like protein
Os07g0160400	-0.549	Glyoxalase bleomycin resistance protein dioxygenase
Os02g0618200	-0.547	Signal transduction response regulator, receiver region domain containing protein
Os12g0288400	-0.547	Retrotransposon ty1-copia subclass
Os07g0173100	-0.546	Hsp20-like chaperone domain containing protein
Os05g0142400	-0.544	Conserved hypothetical protein
Os11g0433600	-0.544	Tetratricopeptide-like helical domain containing protein

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Locus-ID	Log_2FC	Description
Os08g0558200	-0.542	Auxin-regulated protein
Os02g0187100	-0.539	Metal-dependent hydrolase-like protein
Os02g0793700	-0.538	Membrane steroid-binding protein 1
Os07g0619700	-0.537	Hypothetical conserved gene
Os04g0337201	-0.535	Similar to osigba0137o04.8 protein
Os01g0971800	-0.534	Similar to two-component response regulator arr11
Os01g0190000	-0.533	Clavaminate synthase-like protein
Os06g0558766	-0.532	Nuclear poly polymerase
Os03g0807000	-0.531	Conserved hypothetical protein
Os01g0930200	-0.524	Conserved hypothetical protein
Os01g0537250	-0.524	Antigen-like protein
Os01g0856600	-0.523	Structural constituent of nuclear pore
Os07g0479300	-0.522	Carboxypeptidase c cbp31
Os06g0187950	-0.521	Probable cyclic nucleotide-gated ion channel 17-like
Os09g0250700	-0.520	Uncharacterized protein sll1770-like
Os02g0642300	-0.515	Cytochrome b561
Os09g0548400	-0.512	Dimethylaniline monooxygenase, n-oxide-forming domain containing protein
Os01g0155500	-0.511	Receptor-like protein kinase herk 1-like
Os07g0661500	-0.508	Hypothetical conserved gene
Os03g0411300	-0.508	Ef hand family protein
Os03g0265900	-0.508	Conserved hypothetical protein
Os03g0690000	-0.505	Similar to costars family protein
Os04g0194500	-0.503	Abc transporter g family member 28-like
Os07g0283125	-0.503	Receptor-type protein kinase lrk1
Os06g0638200	-0.499	Uncharacterised protein family upf0047 domain containing protein
Os02g0506600	-0.499	Drought-induced protein
Os03g0592500	-0.499	Chlorophyll a b binding protein
Os02g0541325	-0.495	Histidine decarboxylase
Os08g0278900	-0.495	Stromal cell-derived factor 2-like protein
Os06g0694800	-0.493	Protein of unknown function duf1517 domain containing protein
Os03g0128100	-0.490	Callose synthase 3-like
Os02g0301100	-0.488	Bidirectional sugar transporter sweet4-like
Os03g0758100	-0.488	Similar to phosphorylase (fragment)
Os02g0551100	-0.485	Serine threonine-protein kinase
Os05g0539900	-0.485	Uncharacterized protein ycf36-like
Os07g0618700	-0.482	Conserved hypothetical protein
Os12g0256000	-0.479	Probable carboxylesterase 11-like
Os10g0534900	-0.478	Similar to prep (fragment)
Os07g0539700	-0.477	Serine threonine kinase receptor precursor-like protein
Os03g0726200	-0.475	Inositol-tetrakisphosphate 1-kinase 2
Os09g0465600	-0.473	Glucose-6-phosphate isomerase
Os04g0634400	-0.468	Receptor-like serine threonine-protein kinase sd1-8-like
Os09g0471100	-0.466	Similar to peroxidase 17 precursor
Os02g0828200	-0.466	Tpr-like domain containing protein

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Locus-ID	Log_2FC	Description
Os10g0445400	-0.462	Zinc finger, ring/fyve/phd-type domain containing protein
Os02g0180800	-0.462	Non-protein coding transcript
Os06g0316300	-0.461	Glycine rich family protein
Os04g0490500	-0.452	Protein kinase-like domain domain containing protein
Os01g0618100	-0.452	Similar to in2-2 protein
Os05g0475300	-0.451	Vhs domain containing protein
Os11g0533100	-0.448	Similar to card-like transcriptional regulator family protein
Os02g0741900	-0.448	Similar to t23e23.20
Os08g0497600	-0.446	Transducin wd40 domain-containing protein
Os02g0564400	-0.444	Atp-dependent clp protease atp-binding subunit -like
Os05g0579600	-0.442	Homeodomain-like containing protein
Os10g0462600	-0.441	Conserved hypothetical protein
Os02g0437200	-0.438	Snap25 homologous protein snap33
Os11g0116000	-0.437	Nonspecific lipid-transfer protein expressed
Os04g0691100	-0.436	Serine threonine protein kinase sapk4
Os05g0318700	-0.434	Protein kinase
Os12g0164800	-0.433	Hypothetical conserved gene
Os06g0476200	-0.432	Retrotransposon ty1-copia subclass
Os09g0363500	-0.432	Conserved hypothetical protein
Os02g0712000	-0.430	Protease do-like 7-like
Os06g0209300	-0.427	phd finger
Os07g0143200	-0.427	Helix-loop-helix dna-binding domain containing expressed
Os07g0562950	-0.423	Sphingosine kinase
Os01g0303000	-0.420	Calvin cycle protein cp12
Os04g0623100	-0.418	Bromodomain containing protein
Os11g0207200	-0.417	Mitogen activated protein kinase kinase kinase
Os03g0271400	-0.416	Hsp70-binding protein 1-like
Os07g0452500	-0.415	Ormdl family protein
Os02g0816900	-0.410	Myosin xi (fragment)
Os12g0203000	-0.407	Probable serine threonine-protein kinase at1g54610-like
Os07g0150700	-0.405	Cbl-interacting protein kinase 23
Os11g0158832	-0.404	Similar to associated with hox family protein
Os08g0191100	-0.401	Similar to iron-responsive element binding protein
Os12g0507200	-0.400	Similar to eukaryotic translation initiation factor 5a-2 (eif-5a-2)
Os04g0487200	-0.392	Serine/threonine protein kinase domain containing protein
Os09g0442300	-0.392	Cysteine protease
Os05g0156800	-0.390	Conserved hypothetical protein
Os06g0603000	-0.389	Heme oxygenase 1
Os08g0425200	-0.387	Chaperonin-like rbcx domain containing protein
Os04g0620066	-0.380	Abc transporter c family member 2
Os08g0191000	-0.374	Auxin efflux carrier family protein
Os01g0633000	0.373	50s ribosomal protein l31
Os02g0510200	0.373	Acetolactate synthase
Os06g0109000	0.380	Phosphoglycerate mutase domain containing protein

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Locus-ID	Log_2FC	Description
Os04g0539000	0.385	Protein of unknown function duf2485 domain containing protein
Os03g0587000	0.389	Myo-inositol monophosphatase
Os03g0134300	0.391	Retrotransposon ty1-copia subclass
Os03g0234350	0.392	Ubiquitin fusion protein
Os05g0303000	0.393	Stromal 70 kda heat shock-related chloroplastic-like
Os01g0973400	0.393	Radical sam domain-containing protein
Os03g0815400	0.394	Ribosomal protein l17-like protein
Os01g0558300	0.399	Rwd domain containing protein
Os06g0112200	0.403	5 -methylthioadenosine s-adenosylhomocysteine nucleosidase
Os07g0601000	0.403	Nadph hc toxin reductase
Os10g0498300	0.404	Epoxide expressed
Os06g0623300	0.405	Cinnamoyl reductase
Os03g0284400	0.406	50s ribosomal protein l10
Os01g0338600	0.406	Cysteine sulfinate desulfinase cysteine desulfurase and related enzymes
Os08g0515800	0.407	Mitochondrial transcription termination factor family protein
Os10g0502000	0.409	Thylakoid lumenal protein
Os06g0283400	0.411	Probable phosphatidylinositol 4-kinase type 2-beta at1g26270-like
Os11g0544800	0.412	Aspartyl-trna glutamyl-trna amidotransferase subunit b
Os01g0905700	0.413	Ring finger protein
Os03g0452300	0.415	30s ribosomal protein s5
Os10g0445600	0.416	Conserved hypothetical protein
Os09g0505300	0.417	Fata acyl-acp thioesterase
Os03g0219900	0.417	Similar to 50s ribosomal protein l15, chloroplast precursor (cl15) (fragment) k02876 large subunit ribosomal protein l15
Os12g0480100	0.417	One helix protein
Os03g0769100	0.418	Ribosomal protein s9
Os06g0715200	0.420	Conserved hypothetical protein
Os03g0100200	0.422	Transcriptional coactivator/pterin dehydratase family protein
Os05g0497675	0.422	Ribosomal protein l11 methyltransferase
Os09g0460500	0.422	Gibberellin receptor gid1l2
Os02g0822600	0.425	50s ribosomal protein l9
Os03g0595300	0.427	Conserved hypothetical protein
Os04g0412500	0.427	Carbonic anhydrase
Os01g0144100	0.430	Thylakoid lumenal protein 1
Os03g0125600	0.434	Probable serine threonine-protein kinase at1g01540-like
Os03g0135300	0.435	Similar to glutathione s-transferase gst 10
Os03g0758900	0.436	Similar to predicted protein
Os01g0662300	0.437	Ribosomal protein l12
Os05g0490800	0.437	Similar to proteasome subunit alpha type
Os06g0683200	0.437	50s ribosomal protein chloroplast precursor
Os02g0784900	0.438	Rmlc-like jelly roll fold domain containing protein
Os07g0158300	0.438	RNA-binding protein cp33
Os02g0138600	0.442	Protein of unknown function duf1677
Os12g0175500	0.444	Monothiol glutaredoxin-s16

... continuation

Locus-ID	Log_2FC	Description
Os03g0196800	0.444	Esterase lipase thioesterase family
Os10g0547900	0.444	Short chain dehydrogenase reductase family expressed
Os02g0804400	0.444	Conserved hypothetical protein
Os07g0189700	0.445	Similar to jhl07k02.7 protein
Os01g0212400	0.446	Drought-induced protein rdi
Os01g0802100	0.447	Similar to 4-diphosphocytidyl-2-c-methyl-d-erythritol kinase, chloroplast precursor
Os05g0490900	0.447	Conserved hypothetical protein
Os11g0703900	0.447	Heat shock protein 70
Os07g0133700	0.447	Immunophilin fkbp-type peptidyl-prolyl cis-trans isomerase-like protein
Os01g0337900	0.448	Dihydrolipoyl dehydrogenase
Os05g0198400	0.448	Zinc transporter
Os02g0600200	0.449	Iq calmodulin-binding region domain containing protein
Os06g0134000	0.450	40s ribosomal protein s20
Os08g0524200	0.454	Auxin-induced protein
Os01g0952500	0.456	Response regulator
Os03g0177500	0.457	Elongation factor 1
Os02g0652600	0.459	50s ribosomal protein l19
Os04g0462300	0.460	Conserved hypothetical protein
Os05g0420600	0.460	Cytochrome c
Os05g0337400	0.461	Hypothetical protein osj_18112 [*Oryza sativa japonica* group]
Os11g0526200	0.461	Cofactor assembly of complex c
Os02g0833100	0.461	Mate efflux family protein chloroplastic-like
Os08g0558000	0.462	Similar to ribosomal protein
Os04g0438300	0.464	Uncharacterised protein family upf0090 domain containing protein
Os11g0220800	0.465	Similar to 60s ribosomal protein l10 (qm protein homolog)
Os02g0606000	0.467	Glycine-rich protein
Os01g0805000	0.467	50s ribosomal protein l34
Os04g0461100	0.467	Plastid-specific 30s ribosomal protein 3
Os01g0896800	0.468	Ribosomal protein l18/l5 domain containing protein
Os02g0798200	0.469	Ring-h2 zinc finger
Os03g0756000	0.471	Similar to 60s ribosomal protein l13a-4
Os12g0534200	0.472	Acyl carrier protein 3
Os02g0750400	0.472	Pentatricopeptide repeat-containing protein
Os03g0699300	0.472	Adenylosuccinate chloroplast expressed
Os07g0662500	0.473	Elongation factor 1-beta
Os07g0523100	0.474	60s ribosomal protein l44
Os07g0510100	0.475	Methionine aminopeptidase 1b
Os02g0503400	0.476	Similar to 60s ribosomal protein l35
Os09g0551600	0.477	Similar to hmgd1 protein
Os03g0745550	0.477	Conserved hypothetical protein
Os02g0754300	0.479	50s ribosomal protein l29
Os07g0139600	0.479	Non-protein coding transcript
Os03g0122200	0.480	50s ribosomal protein l11
Os03g0781000	0.480	Gtp-dependent nucleic acid-binding protein engd-like

... continuation

Locus-ID	Log_2FC	Description
Os04g0508200	0.481	Isocitrate/isopropylmalate dehydrogenase domain containing protein
Os10g0466700	0.482	60s ribosomal protein l23
Os10g0499600	0.482	Similar to predicted protein
Os04g0250700	0.482	Uncharacterised protein family upf0307 domain containing protein
Os01g0511600	0.483	Lrgb-like family protein
Os02g0220400	0.483	Gata transcription factor, chloroplast development, plant architecture
Os01g0266000	0.485	La rna-binding protein
Os06g0729650	0.486	Photosystem ii stability assembly factor hcf136
Os02g0831200	0.487	Protein of unknown function duf177 family protein
Os06g0555400	0.487	Similar to 40s ribosomal protein s19-like
Os09g0556500	0.488	Cysteinyl-trna synthetase
Os08g0561700	0.488	Superoxide dismutase
Os06g0704700	0.489	Nad(p)-binding domain containing protein
Os04g0493100	0.490	Er33 protein
Os12g0541000	0.490	Riboflavin synthase alpha chain
Os04g0118100	0.492	Glutamyl-trna amidotransferase subunit a-like
Os06g0133800	0.493	Transketolase 1
Os02g0614200	0.495	Conserved hypothetical protein
Os02g0821200	0.497	Ribosomal protein l28e domain containing protein
Os07g0109700	0.498	Conserved hypothetical protein
Os03g0263900	0.499	Ef-hand 2 domain containing protein
Os01g0938100	0.500	Photosystem ii reaction center psb28 protein
Os07g0160300	0.505	Shikimate kinase domain containing protein
Os03g0794700	0.505	Ribosomal protein s18
Os08g0116500	0.505	Similar to 60s acidic ribosomal protein p1
Os06g0332800	0.507	Bundle sheath defective protein
Os08g0433200	0.508	Conserved hypothetical protein
Os11g0158400	0.508	Similar to digalactosyldiacylglycerol synthase 1
Os08g0156800	0.508	60s ribosomal protein l34
Os01g0574600	0.513	Oxidoreductase glyr1-like
Os03g0563300	0.514	Magnesium-chelatase subunit chloroplastic-like
Os04g0476800	0.516	Similar to ta5 protein (fragment)
Os01g0127300	0.516	Sufbd family protein.; k09015 Fe-s cluster assembly protein sufd
Os12g0618800	0.516	Protein of unknown function duf266
Os03g0333400	0.517	Photosystem ii 11 kd protein
Os03g0704000	0.518	30s ribosomal protein s13
Os03g0356300	0.519	50s ribosomal protein l6
Os12g0209100	0.520	Pinus taeda anonymous locus cl2503contig1_03 genomic sequence
Os01g0742000	0.520	Trna rRNA
Os03g0376600	0.521	RNA-binding protein
Os09g0378300	0.522	Similar to leucyl-trna synthetase
Os12g0527301	0.522	Hypothetical gene
Os02g0162500	0.522	Similar to 40s ribosomal protein s14
Os07g0546700	0.524	Similar to 60s ribosomal protein l38

... continuation

Locus-ID	Log_2FC	Description
Os03g0408300	0.524	Similar to 60s ribosomal protein l27a-3
Os01g0739500	0.525	Conserved hypothetical protein
Os09g0252000	0.526	Exosome component 4
Os01g0281000	0.526	F-box protein at2g32560-like
Os01g0279100	0.528	Magnesium-protoporphyrin ix monomethyl ester
Os10g0364600	0.537	Non-protein coding transcript
Os02g0802200	0.539	Heparanase-like protein 3-like
Os03g0234200	0.540	Similar to ubiquitin fusion protein (fragment)
Os04g0602100	0.541	Thylakoid lumenal 29 kda chloroplastic-like
Os03g0315800	0.545	30s ribosomal protein chloroplastic-like
Os04g0303900	0.547	Aminoacyl trna synthase complex-interacting multifunctional protein 1-like
Os03g0807800	0.547	Similar to 40s ribosomal protein s2 (fragment)
Os10g0170200	0.549	40s ribosomal protein s20
Os02g0137200	0.551	50s ribosomal protein l3
Os05g0541900	0.551	Similar to 60s ribosomal protein l28
Os02g0709900	0.551	Nuclear matrix constituent protein 1-like
Os03g0781501	0.552	Non-protein coding transcript
Os07g0677400	0.552	Root peroxidase
Os02g0699600	0.553	Similar to 60s ribosomal protein l12
Os03g0343400	0.553	Similar to photolyase/blue-light receptor phr2
Os11g0601700	0.554	Helix-loop-helix DNA-binding domain containing expressed
Os05g0585900	0.554	Mitochondrial substrate carrier family protein b-like
Os06g0132400	0.555	Magnesium-protoporphyrin ix methyltransferase
Os07g0180900	0.557	60s ribosomal protein l4-1
Os03g0190100	0.557	Ubia prenyltransferase family protein
Os04g0456700	0.559	Elicitor-inducible protein eig-j7
Os08g0148600	0.568	Endoribonuclease dicer homolog 3a-like
Os12g0124200	0.570	Similar to 40s ribosomal protein s16
Os09g0116900	0.570	Conserved hypothetical protein
Os09g0327400	0.572	Aldose 1-epimerase family protein
Os08g0440900	0.572	Chloroplast omega-6 fatty acid desaturase
Os08g0504500	0.575	Thylakoid lumenal 19 kda protein
Os09g0380200	0.575	Dnaj domain containing protein
Os01g0896700	0.578	Similar to 60s ribosomal protein l5
Os01g0662700	0.580	Naphthoate synthase
Os02g0533800	0.582	Conserved hypothetical protein
Os02g0125700	0.583	Lil3 protein
Os08g0308700	0.587	Dcl protein
Os01g0638600	0.587	Glucosyltransferase is10a-like
Os05g0477300	0.590	40s ribosomal protein s26
Os03g0730000	0.591	Lecithin-cholesterol acyltransferase-like 1-like
Os05g0388600	0.592	Protein of unknown function duf3411 domain containing protein
Os12g0167900	0.593	Ribosomal protein l3
Os01g0111100	0.593	Peptidyl-prolyl cis-trans isomerase

... continuation

Locus-ID	Log_2FC	Description
Os02g0814700	0.594	60s ribosomal protein l23
Os02g0192700	0.600	Peroxiredoxin-like protein
Os02g0665250	0.603	Conserved hypothetical protein
Os08g0559200	0.604	Similar to ribosomal protein s25 (40s ribosomal 25s subunit)
Os05g0573700	0.605	Ketol-acid reductoisomerase
Os09g0258600	0.605	Similar to 60s ribosomal protein l17-1
Os03g0350300	0.606	Probable nucleolar protein 5-2-like
Os03g0736600	0.606	Similar to atp synthase
Os09g0530500	0.607	Hypothetical conserved gene
Os08g0234000	0.609	60s ribosomal protein l7-1
Os05g0445500	0.609	60s acidic ribosomal protein p2b
Os01g0328500	0.610	Bucentaur or craniofacial development family protein
Os03g0776900	0.614	Mitochondrial import inner membrane translocase subunit tim14
Os06g0593800	0.615	Udp-glucuronosyl/udp-glucosyltransferase family protein
Os11g0644600	0.615	Conserved hypothetical protein
Os06g0187500	0.616	Hypothetical conserved gene
Os03g0337800	0.617	60s ribosomal protein l19-3
Os03g0299900	0.617	Ll-diaminopimelate chloroplastic-like
Os07g0181500	0.618	Protein of unknown function duf506
Os02g0804500	0.618	Molecular chaperone hsp40 -like protein
Os09g0507800	0.621	60s ribosomal protein l7a
Os02g0321900	0.622	Similar to ribosomal protein l1
Os04g0608600	0.623	Nucleoredoxin 3
Os03g0812000	0.625	Dna gyrase subunit a
Os08g0505300	0.626	Similar to 60s ribosomal protein l31
Os01g0193600	0.630	Rna methyltransferase family protein
Os07g0467200	0.631	Unknown protein [*Oryza sativa japonica* group]
Os04g0175600	0.632	Probable inactive methyltransferase Os04g0175900
Os07g0674200	0.633	Similar to 60s ribosomal protein l22-2
Os05g0459900	0.634	Similar to 60s ribosomal protein l36-1
Os04g0523000	0.634	Oxygen-evolving enhancer protein 3- chloroplastic-like
Os01g0191100	0.635	Similar to acidic ribosomal protein p2a-4
Os02g0704800	0.637	Ornithine carbamoyltransferase
Os08g0542100	0.637	Similar to 60s ribosomal protein l7
Os09g0474300	0.638	Chaperone protein htpg family protein
Os01g0621300	0.647	Hypothetical conserved gene
Os08g0531300	0.647	Translation factor-like
Os08g0326400	0.647	60s ribosomal protein l7a
Os05g0552300	0.648	Guanine nucleotide-binding protein subunit beta-like protein a-like
Os07g0693000	0.649	Similar to ser-thr protein kinase
Os03g0564200	0.649	Protein of unknown function duf952 family protein
Os12g0597400	0.653	Fad nad -binding oxidoreductase domain-containing protein
Os08g0440800	0.655	Nadp-dependent glyceraldehyde-3-phosphate dehydrogenase
Os03g0761000	0.656	Brg-1 associated

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Locus-ID	Log_2FC	Description
Os06g0225900	0.661	P-loop containing nucleoside triphosphate hydrolase domain-containing protein
Os09g0382400	0.663	Conserved hypothetical protein
Os03g0179000	0.664	Tubulin-tyrosine ligase
Os01g0742100	0.665	Trna rRNA
Os03g0841700	0.666	Similar to prohibitin
Os03g0255500	0.670	Similar to phosphoenolpyruvate carboxykinase 4
Os05g0147400	0.672	T-complex protein 1 subunit zeta
Os10g0566700	0.673	Chaperonin cpn10 family protein
Os03g0826500	0.673	Anthranilate synthase alpha 1
Os02g0682600	0.675	Mitochondrial import inner membrane translocase subunit tim13
Os03g0425000	0.676	Similar to predicted protein
Os07g0638100	0.677	Protein tolb
Os05g0593000	0.679	Phospholipase a2, active site domain containing protein
Os02g0714200	0.680	Pyrophosphate--fructose 6-phosphate 1-phosphotransferase alpha subunit
Os03g0126000	0.682	Anthranilate phosphoribosyltransferase
Os02g0159700	0.683	Protein sco1 homolog mitochondrial-like
Os12g0592200	0.684	Conserved hypothetical protein
Os06g0145800	0.685	Dna-binding protein p24
Os07g0109500	0.687	Ribosomal protein l13 family protein
Os08g0529100	0.687	Proteasome subunit beta type 1
Os08g0104600	0.691	Ferredoxin I, chloroplast precursor (anti-disease protein 1)
Os08g0127600	0.692	Heat shock protein dnaj
Os07g0567400	0.694	Similar to cytochrome c6
Os01g0834500	0.697	Similar to 40s ribosomal protein s23 (s12)
Os01g0100700	0.699	40s ribosomal protein s5
Os03g0834050	0.701	Similar to predicted protein
Os01g0348700	0.702	60s ribosomal protein l23a
Os01g0841600	0.703	Triosephosphate isomerase
Os07g0534000	0.708	Permeases of the major facilitator superfamily
Os01g0535900	0.710	Trna (guanine-n -)-methyltransferase-like
Os04g0605900	0.712	60s ribosomal protein l7-1
Os12g0586300	0.712	Myb-like dna-binding domain, shaqkyf class domain containing protein
Os03g0563100	0.712	L-gulonolactone oxidase-like
Os03g0332700	0.712	Abc transporter atpase
Os12g0406200	0.713	40s ribosomal protein s3a
Os07g0614500	0.713	Elongation factor 1-delta 1
Os09g0297400	0.715	Triose phosphate phosphate non-green precursor
Os01g0823300	0.716	Similar to ribosomal protein s26
Os07g0687500	0.717	Peptidyl-prolyl cis-trans isomerase c
Os05g0528900	0.720	Ribosomal protein l9 family protein
Os02g0189000	0.721	Similar to chloroplast 30s ribosomal protein s21
Os03g0831500	0.722	Phosphoribosylformylglycinamidine cyclo-ligase
Os03g0713200	0.723	Conserved hypothetical protein
Os07g0520800	0.724	Ankyrin repeat family protein

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Locus-ID	Log_2FC	Description
Os04g0413600	0.726	Similar to 40s ribosomal protein s14
Os02g0788500	0.726	Nuclear transport factor 2 -like protein
Os01g0860300	0.732	Similar to ribosomal protein l1
Os06g0320200	0.733	Similar to osigba0135c13.7 protein
Os04g0690800	0.735	Chloroplast chlorophyll a b-binding protein
Os02g0777800	0.736	Protein kinase, catalytic domain domain containing protein
Os01g0642900	0.736	Single-stranded dna-binding protein
Os07g0448800	0.739	Aquaporin
Os03g0721300	0.747	Protein of unknown function duf946
Os03g0762900	0.748	Ac090713_7 proteophosphoglycan
Os10g0442100	0.748	3-phosphoglycerate kinase
Os02g0591700	0.764	60s ribosomal protein l6
Os07g0622100	0.766	Similar to ribosomal protein s6 rps6-2
Os02g0612000	0.766	Protein grpe
Os04g0598200	0.785	Similar to 60s ribosomal protein l12
Os12g0516000	0.789	Conserved hypothetical protein
Os07g0191000	0.797	Inositol monophosphatase - like protein
Os05g0215000	0.802	Dehydration-responsive contains burp pf03181
Os11g0521500	0.806	Acyl carrier protein
Os03g0424500	0.810	40s ribosomal protein s19
Os05g0568300	0.813	Ribosomal protein l12
Os02g0749300	0.815	Shikimate kinase
Os02g0815600	0.825	Diphthamide synthesis, dhp1 domain containing protein
Os03g0332600	0.827	Non-protein coding transcript
Os05g0360400	0.839	Zinc c3hc4 type family protein
Os04g0660500	0.841	Serine threonine-protein kinase sepa-like
Os12g0459300	0.843	Helicase-like protein
Os01g0899500	0.846	Hypothetical conserved gene
Os02g0567900	0.856	Similar to h0818e04.14 protein
Os03g0760700	0.862	Aspartate-semialdehyde dehydrogenase-like
Os01g0205200	0.869	Pentatricopeptide repeat-containing protein
Os07g0434500	0.876	Snf2 domain helicase domain-containing protein
Os02g0285800	0.881	Elongation factor family protein
Os02g0202200	0.882	Spx domain-containing protein 1-like
Os03g0337600	0.883	Uroporphyrinogen decarboxylase
Os03g0751400	0.886	50s ribosomal protein l6
Os08g0269700	0.892	Conserved hypothetical protein
Os09g0485900	0.893	Similar to 60s ribosomal protein l9
Os02g0762100	0.911	Similar to regulator of ribonuclease-like protein 2
Os01g0757900	0.948	Haloacid dehalogenase/epoxide hydrolase domain containing protein
Os09g0277800	0.952	Enoyl-acp reductase
Os02g0229000	0.956	Similar to 40s ribosomal protein s19-like
Os12g0438600	0.967	Chloride channel
Os01g0940000	0.973	Cytokinin dehydrogenase

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Locus-ID	Log_2FC	Description
Os03g0246500	0.985	Protein of unknown function duf869
Os01g0844300	0.992	Peptidylprolyl cis-trans isomerase
Os08g0240000	0.998	Flavonol 3-sulfotransferase
Os10g0529300	1.012	Glutathione s-transferase
Os04g0161100	1.017	Similar to osigba0102o13.5 protein
Os08g0469700	1.045	Phosphatidylinositol-4-phosphate 5-kinase
Os02g0755600	1.089	Udp-glucuronosyl/udp-glucosyltransferase domain containing protein
Os01g0631700	1.110	Similar to ser thr specific protein kinase-like protein
Os06g0217700	1.111	Threonine endopeptidase
Os01g0118400	1.120	Similar to hga6
Os02g0131050	1.120	Hypothetical gene
Os07g0616600	1.164	40s ribosomal protein
Os10g0355800	1.273	Atp synthase cf1 beta subunit
Os05g0363500	1.318	Transducin wd-40 repeat-containing protein
Os10g0356000	1.322	Ribulose- -bisphosphate carboxylase oxygenase large subunit
Os03g0764600	1.391	Homeodomain-like containing protein
Os01g0284700	1.393	Peptidyl-prolyl cis-trans isomerase
Os03g0399800	1.550	Mannose-binding lectin domain containing protein
Os10g0214132	1.634	Ribosomal protein l2
Os09g0537700	1.797	Rnase s-like protein precursor
Os05g0227600	1.906	Proline-rich glycoprotein, aba-dependent inhibition of root growth
Os12g0106000	2.174	Similar to ferritin 1, chloroplast precursor
Os03g0251000	2.901	Plant lipid transfer protein/seed storage/trypsin-alpha amylase inhibitor domain containing protein
Os09g0396900	3.594	Vacuolar iron transporter

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3109 **Anexo B – Additional file 2 (Artigo 2)**

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Cellular component	Up regulated	Down regulated
Mitochondrion	57	39
Plastid	54	39
Cytoplasmic membrane-bounded vesicle	23	48
Membrane	23	35
Chloroplast stroma	37	6
Nucleus	16	17
Chloroplast envelope	25	4
Cytosol	15	10
Chloroplast	23	0
Ribosome	22	0
Chloroplast	8	12
Plasma membrane	8	8
Chloroplast thylakoid membrane	13	2
Cytoplasm	3	7
Nucleolus	8	1
Chloroplast thylakoid lumen	7	0
Plasmodesma	6	1
Vacuolar membrane	5	2
Thylakoid	5	1
Transcription factor complex	3	3
Extracellular region	1	5
Cytosolic large ribosomal subunit	5	0
Cell wall	3	1
Golgi apparatus	2	2
Vacuole	1	3
Cytosolic small ribosomal subunit	3	0
Apoplast	2	1
Chloroplast thylakoid	1	2
Peroxisome	1	2
Plant-type vacuole membrane	1	2
Intracellular membrane-bounded organelle	0	3
Chloroplast photosystem II	2	0
Eukaryotic translation elongation factor 1 complex	2	0
Nucleoid	2	0
Plastid chromosome	2	0
Plastid large ribosomal subunit	2	0
Small ribosomal subunit	2	0
DNA polymerase complex	1	1
Heterotrimeric G-protein complex	1	1
Mitochondrial inner membrane	1	1
Plant-type vacuole	1	1
Cytoplasmic part	0	2

... continuation

Cellular component	Up regulated	Down regulated
Endoplasmic reticulum	0	2
Integral component of membrane	0	2
Intracellular part	0	2
90S preribosome	1	0
Beta-galactosidase complex	1	0
Chloroplast inner membrane	1	0
Chloroplast part	1	0
Chloroplast ribulose bisphosphate carboxylase complex	1	0
Chloroplast stromal thylakoid	1	0
Chloroplast thylakoid	1	0
Cytosolic ribosome	1	0
Fatty acid synthase complex	1	0
Large ribosomal subunit	1	0
Mediator complex	1	0
Mitochondrial large ribosomal subunit	1	0
Mitochondrial respiratory chain	1	0
NAD(P)H dehydrogenase complex (plastoquinone)	1	0
Nuclear lumen	1	0
Nuclear membrane	1	0
Photosystem II reaction center	1	0
Plant-type cell wall	1	0
Plastid stroma	1	0
Proteasome core complex	1	0
Proton-transporting two-sector atpase complex	1	0
PSII associated light-harvesting complex II	1	0
Riboflavin synthase complex	1	0
Sarcoplasmic reticulum	1	0
Stromule	1	0
Cell part	0	1
Cell plate	0	1
Chloroplast membrane	0	1
Cul4-RING E3 ubiquitin ligase complex	0	1
Cytosol mitochondrion	0	1
Endoplasmic reticulum membrane	0	1
Filiform apparatus	0	1
Glycine cleavage complex	0	1
Glycolate oxidase complex	0	1
Integral component of plasma membrane	0	1
Integrin complex	0	1
Intracellular	0	1
Nuclear envelope	0	1
Plastoglobule	0	1
Trans-Golgi network	0	1

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Molecular function	Up regulated	Down regulated
Structural constituent of ribosome	30	0
Protein binding	9	7
ATP binding	9	6
DNA binding	5	4
RNA binding	6	1
Copper ion binding	5	0
Protein serine/threonine kinase activity	1	4
Translation elongation factor activity	4	0
Sequence-specific DNA binding transcription factor activity	2	2
Peptidyl-prolyl cis-trans isomerase activity	3	0
Oxidoreductase activity	2	1
Zinc ion binding	2	1
Calcium ion binding	1	2
Protein tyrosine kinase activity	1	2
FK506 binding	2	0
GTP binding	2	0
5'-3' exonuclease activity	1	1
Atpase activity	1	1
Atpase activity, coupled to transmembrane movement of substances	1	1
Calmodulin binding	1	1
Catalytic activity	1	1
Flavin adenine dinucleotide binding	1	1
Methionine adenosyltransferase activity	1	1
Peroxidase activity	1	1
Ribulose-phosphate 3-epimerase activity	1	1
Transcription factor binding	1	1
DNA-directed RNA polymerase activity	0	2
Oxidoreductase activity, acting on a sulfur group of donors	0	2
Transporter activity	0	2
1,4-dihydroxy-2-naphthoyl-coa synthase activity	1	0
3-chloroallyl aldehyde dehydrogenase activity	1	0
ACP phosphopantetheine attachment site binding involved in fatty acid biosynthetic process	1	0
Acyl-[acyl-carrier-protein] hydrolase activity	1	0
Amidase activity	1	0
Anion channel activity	1	0
Anthranilate phosphoribosyltransferase activity	1	0
Antioxidant activity	1	0
Aspartate-semialdehyde dehydrogenase activity	1	0
Beta-D-fucosidase activity	1	0
Beta-galactosidase activity	1	0
Beta-gentiobiose beta-glucosidase activity	1	0

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Molecular function	Up regulated	Down regulated
Beta-L-arabinosidase activity	1	0
Calcium:sodium antiporter activity	1	0
Calcium-transporting atpase activity	1	0
Carbonate dehydratase activity	1	0
Chlorophyll binding	1	0
Cysteine-trna ligase activity	1	0
Cytokinin dehydrogenase activity	1	0
DNA topoisomerase activity	1	0
DNA-dependent atpase activity	1	0
DNA-directed DNA polymerase activity	1	0
Double-stranded telomeric DNA binding	1	0
Electron carrier activity	1	0
Electron transporter	1	0
Glucan exo-1,3-beta-glucosidase activity	1	0
Glutaminyl-trna synthase (glutamine-hydrolyzing) activity	1	0
Glutathione transferase activity	1	0
Glyceraldehyde-3-phosphate dehydrogenase ($NADP^+$) (non-phosphorylating) activity	1	0
Glyoxylate reductase (NADP) activity	1	0
Hydrolase activity	1	0
Indoleacetamide hydrolase activity	1	0
Iron ion transmembrane transporter activity	1	0
Kinase activity	1	0
L,L-diaminopimelate aminotransferase activity	1	0
L-ascorbate peroxidase activity	1	0
Magnesium ion binding	1	0
Magnesium protoporphyrin IX methyltransferase activity	1	0
Metalloexopeptidase activity	1	0
Methylthioadenosine nucleosidase activity	1	0
NADPH binding	1	0
Nitrate:proton symporter activity	1	0
Nucleotide binding	1	0
Omega-6 fatty acid desaturase activity	1	0
Oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	1	0
Phosphatase activity	1	0
Phosphoenolpyruvate:phosphate antiporter activity	1	0
Phosphogluconate dehydrogenase (decarboxylating) activity	1	0
Phosphoribosylformylglycinamidine cyclo-ligase activity	1	0
Poly(U) RNA binding	1	0
P-P-bond-hydrolysis-driven protein transmembrane transporter activity	1	0
Protein homodimerization activity	1	0
Riboflavin synthase activity	1	0

... continuation

Molecular function	Up regulated	Down regulated
Ribulose-bisphosphate carboxylase activity	1	0
RNA methyltransferase activity	1	0
Sequence-specific DNA binding	1	0
Shikimate kinase activity	1	0
Sugar:proton symporter activity	1	0
Superoxide dismutase activity	1	0
Transferase activity, transferring hexosyl groups	1	0
Transketolase activity	1	0
Triose-phosphate isomerase activity	1	0
Triose-phosphate:phosphate antiporter activity	1	0
Trna binding	1	0
Tubulin-tyrosine ligase activity	1	0
UDP-glycosyltransferase activity	1	0
Unfolded protein binding	1	0
Uroporphyrinogen decarboxylase activity	1	0
Xanthophyll binding	1	0
3-deoxy-7-phosphoheptulonate synthase activity	0	1
ARF guanyl-nucleotide exchange factor activity	0	1
ATP-dependent helicase activity	0	1
Auxin:proton symporter activity	0	1
Carboxy-lyase activity	0	1
Cysteine-type endopeptidase activity	0	1
D-erythro-sphingosine kinase activity	0	1
Endonuclease activity	0	1
FAD binding	0	1
Glycine dehydrogenase (decarboxylating) activity	0	1
Glycolate oxidase activity	0	1
Heme binding	0	1
Heme oxygenase (decyclizing) activity	0	1
Indole-3-acetaldehyde oxidase activity	0	1
Molybdate ion transmembrane transporter activity	0	1
Molybdopterin cofactor binding	0	1
Nicotianamine aminotransferase activity	0	1
Nucleotide kinase activity	0	1
Nucleotidyltransferase activity	0	1
Phosphatidic acid binding	0	1
Phosphoacetylglucosamine mutase activity	0	1
Phosphoethanolamine N-methyltransferase activity	0	1
Plastid sigma factor activity	0	1
Poly(A) binding	0	1
Polyamine oxidase activity	0	1
Primary amine oxidase activity	0	1
RNA-directed DNA polymerase activity	0	1
Serine-type carboxypeptidase activity	0	1

... continuation

Molecular function	Up regulated	Down regulated
Serine-type endopeptidase activity	0	1
Serine-type endopeptidase inhibitor activity	0	1
Serine-type peptidase activity	0	1
SNAP receptor activity	0	1
Sphinganine kinase activity	0	1
Sugar transmembrane transporter activity	0	1
Sulfate transmembrane transporter activity	0	1
Zeaxanthin epoxidase [overall] activity	0	1
Zeaxanthin epoxidase activity	0	1
Zinc ion transmembrane transporter activity	0	1

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Biological process	Up regulated	Down regulated
Translation	27	0
Ribosome biogenesis	25	0
Isopentenyl diphosphate biosynthetic process	23	2
rRNA processing	16	0
Thylakoid membrane organization	16	0
Chlorophyll biosynthetic process	13	1
Pentose-phosphate shunt	12	2
Photosystem II assembly	13	0
Embryo development ending in seed dormancy	10	0
Response to cadmium ion	6	3
Chloroplast relocation	8	0
Ovule development	7	0
Positive regulation of transcription, DNA-templated	7	0
Regulation of protein dephosphorylation	7	0
Carotenoid biosynthetic process	6	1
Defense response to bacterium	5	2
Response to cold	4	3
Starch biosynthetic process	4	3
Response to salt stress	3	4
Iron-sulfur cluster assembly	6	0
RNA methylation	6	0
Transcription from plastid promoter	6	0
Stomatal complex morphogenesis	5	1
Maltose metabolic process	3	3
Proteolysis	1	5
Response to abscisic acid	1	5
Chloroplast organization	5	0
mRNA modification	5	0
Protein folding	5	0

... continuation

Biological process	Up regulated	Down regulated
Phosphatidylglycerol biosynthetic process	4	1
Positive regulation of catalytic activity	4	1
Oxidation-reduction process	2	3
Serine family amino acid metabolic process	1	4
DNA-templated transcription, elongation	4	0
Pyrimidine ribonucleotide biosynthetic process	4	0
Regulation of translational elongation	4	0
Vegetative to reproductive phase transition of meristem	4	0
Cellular cation homeostasis	3	1
Cysteine biosynthetic process	3	1
Divalent metal ion transport	3	1
Hydrogen peroxide catabolic process	3	1
Leaf morphogenesis	3	1
Ubiquinone biosynthetic process	3	1
Unsaturated fatty acid biosynthetic process	3	1
Photosynthesis	2	2
Regulation of transcription, DNA-templated	2	2
Transport	2	2
Golgi organization	1	3
Jasmonic acid biosynthetic process	1	3
Protein phosphorylation	1	3
Cellular metabolic process	3	0
Glucosinolate biosynthetic process	3	0
Indoleacetic acid biosynthetic process	3	0
Photosynthetic electron transport in photosystem I	3	0
Protein targeting to chloroplast	3	0
Purine nucleotide biosynthetic process	3	0
Response to blue light	3	0
Response to far red light	3	0
Response to red light	3	0
Aromatic amino acid family biosynthetic process	2	1
Carbon utilization	2	1
Cation transport	2	1
Cell differentiation	2	1
L-phenylalanine biosynthetic process	2	1
Response to oxidative stress	2	1
Tryptophan biosynthetic process	2	1
Tyrosine biosynthetic process	2	1
Defense response to fungus	1	2
Glycolytic process	1	2
Response to water deprivation	1	2
Response to wounding	0	3
Arginine metabolic process	2	0
Calcium ion transport	2	0

... continuation

Biological process	Up regulated	Down regulated
Electron transport	2	0
Electron transport chain	2	0
Ethylene biosynthetic process	2	0
Gluconeogenesis	2	0
Glyoxylate metabolic process	2	0
Metabolic process	2	0
Methylglyoxal catabolic process to D-lactate	2	0
Negative regulation of transcription, DNA-templated	2	0
Oligopeptide transport	2	0
Peroxidase reaction	2	0
Photosynthesis, light reaction	2	0
Plant-type cell wall organization	2	0
Plastid organization	2	0
Production of miRNAs involved in gene silencing by miRNA	2	0
Production of ta-sirnas involved in RNA interference	2	0
Proline metabolic process	2	0
Protein peptidyl-prolyl isomerization	2	0
Regulation of proton transport	2	0
Response to heat	2	0
Response to karrikin	2	0
Response to stimulus	2	0
Response to zinc ion	2	0
Single-organism metabolic process	2	0
Tryptophan catabolic process	2	0
Ubiquitin-dependent protein catabolic process	2	0
Cell wall modification	1	1
Cellular aromatic compound metabolic process	1	1
Cellular response to abscisic acid stimulus	1	1
Coenzyme biosynthetic process	1	1
Determination of bilateral symmetry	1	1
DNA recombination	1	1
DNA replication	1	1
Glycosphingolipid metabolic process	1	1
Iron ion transport	1	1
Lipoate metabolic process	1	1
L-serine metabolic process	1	1
Meristem initiation	1	1
Methionine metabolic process	1	1
Protein autophosphorylation	1	1
PSII associated light-harvesting complex II catabolic process	1	1
Purine nucleobase metabolic process	1	1
Response to auxin	1	1
Response to cyclopentenone	1	1
Response to jasmonic acid	1	1

... continuation

Biological process	Up regulated	Down regulated
S-adenosylmethionine biosynthetic process	1	1
Salicylic acid biosynthetic process	1	1
Secondary metabolic process	1	1
Seed germination	1	1
Systemic acquired resistance	1	1
Threonine metabolic process	1	1
Tryptophan metabolic process	1	1
Vacuole organization	1	1
Vitamin metabolic process	1	1
Biosynthetic process	0	2
Golgi vesicle transport	0	2
Hyperosmotic response	0	2
Positive regulation of flavonoid biosynthetic process	0	2
Protein targeting to membrane	0	2
Response to fungus	0	2
Response to mechanical stimulus	0	2
Response to temperature stimulus	0	2
Water transport	0	2
Abscisic acid-activated signaling pathway	1	0
Acetyl-coa biosynthetic process from pyruvate	1	0
Acyl-carrier-protein biosynthetic process	1	0
Anion transport	1	0
Anther development	1	0
Aromatic amino acid family metabolic process	1	0
ATP synthesis coupled proton transport	1	0
Benzoate metabolic process	1	0
Biological regulation	1	0
Calcium ion homeostasis	1	0
Carbon fixation	1	0
Cell death	1	0
Cell-cell signaling	1	0
Cellular amino acid biosynthetic process	1	0
Cellular biosynthetic process	1	0
Cellular component organization	1	0
Cellular component organization or biogenesis	1	0
Cellular macromolecule metabolic process	1	0
Cellular process	1	0
Cellular protein modification process	1	0
Cellular response to cold	1	0
Cellular response to phosphate starvation	1	0
Cellular response to salt stress	1	0
Cellular zinc ion homeostasis	1	0
Chlorophyll biosynthetic process photosynthetic electron transport in photosystem I	1	0

... continuation

Biological process	Up regulated	Down regulated
Chloroplast RNA processing	1	0
Chromatin silencing by small RNA	1	0
Chromosome organization	1	0
Circadian rhythm	1	0
Cotyledon vascular tissue pattern formation	1	0
Cristae formation	1	0
Cuticle development	1	0
Cysteine metabolic process	1	0
Cysteinyl-trna aminoacylation	1	0
Cytochrome b6f complex assembly	1	0
Cytoskeleton organization	1	0
De-etiolation	1	0
Defense response	1	0
Defense response to virus	1	0
Defense response, incompatible interaction	1	0
D-gluconate metabolic process	1	0
DNA repair	1	0
DNA topological change	1	0
DNA-dependent DNA replication	1	0
Drug transport	1	0
Endonucleolytic cleavage in ITS1 to separate SSU-rRNA from 5.8S rRNA	1	0
Endonucleolytic cleavage to generate mature 3'-end of SSU-rRNA	1	0
Ethylene-activated signaling pathway	1	0
Fatty acid biosynthetic process	1	0
Flower morphogenesis	1	0
Fructose metabolic process	1	0
Galactolipid biosynthetic process	1	0
Galactose metabolic process	1	0
Gene silencing	1	0
Glucosinolate metabolic process	1	0
Glutamate metabolic process	1	0
Glutamyl-trna aminoacylation	1	0
Glutathione conjugation reaction	1	0
Glutathione metabolic process	1	0
Glycerolipid metabolic process	1	0
Glycine catabolic process	1	0
Glycine metabolic process	1	0
Glycosaminoglycan catabolic process	1	0
Granum assembly	1	0
Histone H3-K9 methylation	1	0
Inositol metabolic process	1	0
Intracellular sequestering of iron ion	1	0
Intracellular signal transduction	1	0

... continuation

Biological process	Up regulated	Down regulated
L-ascorbic acid metabolic process	1	0
Leaf development	1	0
Leaf vascular tissue pattern formation	1	0
L-methionine biosynthetic process from S-adenosylmethionine	1	0
L-phenylalanine metabolic process	1	0
Lysine biosynthetic process	1	0
Lysine biosynthetic process via diaminopimelate	1	0
Mannose metabolic process	1	0
Meiotic nuclear division	1	0
Meristem maintenance	1	0
Methylation-dependent chromatin silencing	1	0
NADH dehydrogenase complex (plastoquinone) assembly	1	0
Ncrna metabolic process	1	0
Negative regulation of DNA recombination	1	0
Nitrogen compound metabolic process	1	0
Nonphotochemical quenching	1	0
N-terminal protein amino acid modification	1	0
Nucleic acid metabolic process	1	0
One-carbon metabolic process	1	0
Organelle organization	1	0
Organic substance biosynthetic process	1	0
Organic substance metabolic process	1	0
Organonitrogen compound metabolic process	1	0
Oxidoreduction coenzyme metabolic process	1	0
Oxylipin biosynthetic process	1	0
Phloem or xylem histogenesis	1	0
Phosphoenolpyruvate transport	1	0
Phosphoenolpyruvate-dependent sugar phosphotransferase system	1	0
Photosynthetic electron transport chain	1	0
Photosystem II repair	1	0
Plastid translation	1	0
Polar nucleus fusion	1	0
Pollen development	1	0
Positive regulation of cellular response to phosphate starvation	1	0
Positive regulation of respiratory burst	1	0
Primary metabolic process	1	0
Proteasomalprotein catabolicprocess	1	0
Protein import into chloroplast stroma	1	0
Protein transport	1	0
Protein ubiquitination	1	0
Pyruvate metabolic process	1	0
Regulation of cellular process	1	0
Regulation of gene expression, epigenetic	1	0
Regulation of lipid metabolic process	1	0

... continuation

Biological process	Up regulated	Down regulated
Regulation of programmed cell death	1	0
Regulation of timing of transition from vegetative to reproductive phase	1	0
Removal of superoxide radicals	1	0
Response to brassinosteroid	1	0
Response to chlorate	1	0
Response to deep water	1	0
Response to endoplasmic reticulum stress	1	0
Response to ethylene	1	0
Response to gibberellin	1	0
Response to high light intensity	1	0
Response to nematode	1	0
Response to reactive oxygen species	1	0
Response to salicylic acid	1	0
Response to sucrose	1	0
Riboflavin biosynthetic process	1	0
Ribosomal small subunit assembly	1	0
RNA metabolic process	1	0
RNA modification	1	0
RNA processing	1	0
Root development	1	0
rRNA export from nucleus	1	0
rRNA processing thylakoid membrane organization	1	0
Shikimate metabolic process	1	0
Shoot system development	1	0
Single-organism biosynthetic process	1	0
Single-organism cellular process	1	0
Single-organism organelle organization	1	0
Single-organism process	1	0
Small molecule metabolic process	1	0
Starch metabolic process	1	0
Styrene catabolic process	1	0
Sucrose metabolic process	1	0
Sulfur amino acid metabolic process	1	0
Sulfur compound biosynthetic process	1	0
Systemic acquired resistance	1	0
Toxin catabolic process	1	0
Transcription factor import into nucleus	1	0
Triose phosphate transmembrane transport	1	0
Tyrosine metabolic process	1	0
Vegetative phase change	1	0
Very long-chain fatty acid metabolic process	1	0
Virus induced gene silencing	1	0
Vitamin K biosynthetic process	1	0

... continuation

Biological process	Up regulated	Down regulated
Xylem and phloem pattern formation	1	0
Abscisic acid biosynthetic process	0	1
Acetyl-coa metabolic process	0	1
Amino acid import	0	1
Ammonium transport	0	1
Anthocyanin-containing compound biosynthetic process	0	1
Basic amino acid transport	0	1
Calcium-mediated signaling	0	1
Carbohydrate transport	0	1
Cell morphogenesis	0	1
Cell redox homeostasis	0	1
Cell-matrix adhesion	0	1
Cellular amino acid metabolic process	0	1
Cellular response to light stimulus	0	1
Cellular response to UV-C	0	1
Cellular response to water deprivation	0	1
Cellulose biosynthetic process	0	1
Chlorophyll metabolic process	0	1
Chloroplast-nucleus signaling pathway	0	1
Chlororespiration	0	1
Chorismate biosynthetic process	0	1
Cytokinesis by cell plate formation	0	1
Cytokinin biosynthetic process	0	1
Detection of biotic stimulus	0	1
Detection of calcium ion	0	1
DNA-templated transcription, initiation	0	1
Endoplasmic reticulum unfolded protein response	0	1
ER to Golgi vesicle-mediated transport	0	1
Ethanolamine metabolic process	0	1
Glycine decarboxylation via glycine cleavage system	0	1
Heme oxidation	0	1
Inflorescence development	0	1
Jasmonic acid mediated signaling pathway	0	1
MAPK cascade	0	1
Modulation by virus of host morphology or physiology	0	1
Multicellular organismal water homeostasis	0	1
Negative regulation of programmed cell death	0	1
N-terminal protein myristoylation	0	1
Nuclear-transcribed mRNA catabolic process	0	1
Nucleotide transport	0	1
Peroxidase reaction response to oxidative stress	0	1
Photoinhibition	0	1
Photoperiodism, flowering	0	1
Photorespiration	0	1

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Biological process	Up regulated	Down regulated
Phytochromobilin biosynthetic process	0	1
Polarity specification of adaxial/abaxial axis	0	1
Pollen germination	0	1
Pollen tube reception	0	1
Polyamine catabolic process	0	1
Post-embryonic development	0	1
Protein catabolic process	0	1
Protein glycosylation	0	1
Protein targeting to vacuole	0	1
Pyrimidine nucleobase metabolic process	0	1
Red, far-red light phototransduction	0	1
Regulation of gtpase activity	0	1
Regulation of hydrogen peroxide metabolic process	0	1
Regulation of ion transport	0	1
Regulation of meristem growth	0	1
Regulation of multi-organism process	0	1
Regulation of photomorphogenesis	0	1
Regulation of photosynthesis	0	1
Regulation of plant-type hypersensitive response	0	1
Regulation of RNA biosynthetic process	0	1
Respiratory burst involved in defense response	0	1
Response to chitin	0	1
Response to desiccation	0	1
Response to fructose	0	1
Response to insect	0	1
Response to ozone	0	1
RNA polyadenylation	0	1
RNA-dependent DNA replication	0	1
Root hair elongation	0	1
Second-messenger-mediated signaling	0	1
Signal transduction	0	1
Sodium ion transport	0	1
Sulfate transport	0	1
UDP-N-acetylglucosamine biosynthetic process	0	1
Vesicle-mediated transport	0	1
Xanthophyll biosynthetic process	0	1
Zinc ion transport	0	1
Cellular cation homeostasis	0	0

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Anexo C – Additional file 3 (Artigo 2)

Additional file 3. List of possible candidate genes the iron homeostasis in the cell found both in our experiments in comparison the literature.

Metabolism	Gene-ID	log_2FC	Description
Amino acid activation protein	Os09g0556500	0.488	Cysteinyl-tRNA synthetase
Amino acid metabolism	Os02g0306401	-1.441	Nicotianamine aminotransferase
Abiotic stress	Os12g0569700	-0.735	Similar to heat shock protein 70
Abiotic stress	Os02g0833100	0.461	Mate efflux family protein chloroplastic-like
Abiotic stress	Os03g0776900	0.614	Mitochondrial import inner membrane translocase subunit tim14
Amino acid metabolism	Os07g0622200	-0.706	2-dehydro-3-deoxyphosphoheptonate aldolase 3-deoxy-d-arabino-heptulosonate 7-phosphate synthetase
Amino acid metabolism	Os06g0112200	0.403	5 -methylthioadenosine s-adenosylhomocysteine nucleosidase
Amino acid metabolism	Os03g0126000	0.682	Anthranilate phosphoribosyltransferase
Biodegradation of xenobiotics	Os09g0460500	0.422	Gibberellin receptor gid1l2
C1-metabolism	Os02g0762100	0.911	Similar to regulator of ribonuclease-like protein 2
Cell wall	Os08g0434632	-0.797	Glucomannan 4-beta-mannosyltransferase 9-like
Co-factor and vitamine metabolism	Os01g0662700	0.580	Naphthoate synthase
Cycle cell	Os01g0111100	0.593	Peptidyl-prolyl cis-trans isomerase
Degradation protein	Os06g0125800	-0.624	E3 ligase
Degradation protein	Os06g0283400	0.411	Probable phosphatidylinositol 4-kinase type 2-beta at1g26270-like
Degradation protein	Os04g0476800	0.516	Similar to ta5 protein (fragment)
Degradation protein	Os08g0529100	0.687	Proteasome subunit beta type 1
Development	Os02g0301100	-0.488	Bidirectional sugar transporter sweet4-like
DNA	Os11g0533100	-0.448	Similar to card-like transcriptional regulator family protein, expressed
Folding protein	Os05g0147400	0.672	T-complex protein 1 subunit zeta
Folding protein	Os01g0844300	0.992	Peptidylprolyl cis-trans isomerase
Gluconeogenesis	Os08g0434300	-1.035	Nad-malate dehydrogenase
Glycolysis	Os09g0465600	-0.473	Glucose-6-phosphate isomerase
Glycolysis	Os06g0476200	-0.432	Retrotransposon ty1-copia subclass
Hormone metabolism	Os10g0536400	-1.097	Similar to oxidoreductase, 2og-fe oxygenase family protein
Hormone metabolism	Os07g0164900	-0.861	Aldehyde oxidase
Lipid metabolism	Os11g0116000	-0.437	Nonspecific lipid-transfer protein expressed
Major CHO metabolism	Os02g0550600	-0.698	Plant neutral invertase family protein
Metal handling	Os12g0106000	2.174	Similar to ferritin 1, chloroplast precursor
Metal handling	Os09g0396900	3.594	Vacuolar iron transporter
Minor CHO metabolism	Os01g0847700	-0.585	Similar to aldose reductase
Miscellaneous enzyme families	Os03g0140200	-0.960	Cytochrome p450 family expressed

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Metabolism	Gene-ID	log_2FC	Description
Miscellaneous enzyme families	Os01g0138900	-0.975	L-ala-d l-glu epimerase-like
Miscellaneous enzyme families	Os04g0623300	-1.168	Probable polyamine oxidase 2-like
Miscellaneous enzyme families	Os10g0547900	0.444	Short chain dehydrogenase reductase family expressed
Miscellaneous enzyme families	Os04g0531750	-0.938	Salutaridine reductase-like
Miscellaneous enzyme families	Os02g0755600	1.089	Udp-glucuronosyl/udp-glucosyltransferase domain containing protein.
Miscellaneous enzyme families	Os06g0187500	0.616	Hypothetical conserved gene
Not assigned	Os06g0606700	-1.112	Tetratricopeptide-like helical domain containing protein
Not assigned	Os09g0271100	-1.026	Conserved hypothetical protein
Not assigned	Os03g0184100	-0.926	Conserved hypothetical protein
Not assigned	Os12g0601800	-0.896	Similar to bzip transcription factor family protein
Not assigned	Os10g0389500	-0.833	Conserved hypothetical protein
Not assigned	Os01g0665900	-0.815	Conserved hypothetical protein
Not assigned	Os02g0286933	-0.798	Conserved hypothetical protein
Not assigned	Os01g0678100	-0.779	Protein of unknown function duf2921 domain containing protein
Not assigned	Os10g0501500	-0.709	Protein of unknown function duf607 family protein
Not assigned	Os07g0569600	-0.658	Chaperonin-like rbcx domain containing protein
Not assigned	Os07g0660100	-0.642	Similar to predicted protein
Not assigned	Os04g0442800	-0.598	Similar to phragmoplast-associated kinesin-related protein 1
Not assigned	Os03g0168200	-0.594	Similar to f16a14.21
Not assigned	Os02g0506600	-0.499	Drought-induced protein
Not assigned	Os05g0475300	-0.451	Vhs domain containing protein
Not assigned	Os03g0271400	-0.416	Hsp70-binding protein 1-like
Not assigned	Os08g0425200	-0.387	Chaperonin-like rbcx domain containing protein
Not assigned	Os01g0558300	0.399	Rwd domain containing protein
Not assigned	Os01g0338600	0.406	Cysteine sulfinate desulfinase cysteine desulfurase and related enzymes
Not assigned	Os10g0445600	0.416	Conserved hypothetical protein
Not assigned	Os03g0100200	0.422	Transcriptional coactivator/pterin dehydratase family protein.
Not assigned	Os07g0189700	0.445	Similar to jhl07k02.7 protein
Not assigned	Os11g0526200	0.461	Cofactor assembly of complex c
Not assigned	Os02g0798200	0.469	Ring-h2 zinc finger
Not assigned	Os02g0802200	0.539	Heparanase-like protein 3-like
Not assigned	Os08g0148600	0.568	Endoribonuclease dicer homolog 3a-like
Not assigned	Os07g0181500	0.618	Protein of unknown function duf506
Not assigned	Os03g0812000	0.625	Dna gyrase subunit a
Not assigned	Os03g0425000	0.676	Similar to predicted protein
Not assigned	Os07g0520800	0.724	Ankyrin repeat family protein
Not assigned	Os03g0762900	0.748	Ac090713_7 proteophosphoglycan
Not assigned	Os03g0332600	0.827	Non-protein coding transcript
Not assigned	Os02g0131050	1.120	Hypothetical gene

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Metabolism	Gene-ID	log_2FC	Description
Organisation cell	Os03g0179000	0.664	Tubulin-tyrosine ligase
Ortho-phenylphenol	Os01g0574600	0.513	Oxidoreductase glyr1-like
Photosynthesis	Os10g0442100	0.748	3-phosphoglycerate kinase
Photosynthesis	Os10g0356000	1.322	Ribulose- -bisphosphate carboxylase oxygenase large subunit
Photosynthesis	Os07g0567400	0.694	Similar to cytochrome c6
Postranslational modification protein	Os02g0799000	-1.157	Similar to DNA-binding protein phosphatase 2c
Postranslational modification protein	Os01g0655500	-0.990	Protein kinase, core domain containing protein
Postranslational modification protein	Os07g0283125	-0.503	Receptor-type protein kinase lrk1
Postranslational modification protein	Os04g0490500	-0.452	Protein kinase-like domain domain containing protein
Postranslational modification protein	Os04g0691100	-0.436	Serine threonine protein kinase sapk4
Processing RNA	Os09g0537700	1.797	Rnase s-like protein precursor
Redox	Os04g0602100	0.541	Thylakoid lumenal 29 kda chloroplastic-like
Redox	Os08g0561700	0.488	Superoxide dismutase
Regulation of transcription	Os08g0515800	0.407	Mitochondrial transcription termination factor family protein
Regulation of transcription	Os05g0183100	-0.630	Similar to wrky transcription factor 16 (fragment)
RNA binding	Os07g0158300	0.438	RNA-binding protein cp33
Synthesis protein	Os07g0614500	0.713	Elongation factor 1-delta 1
Synthesis protein	Os03g0452300	0.415	30s ribosomal protein s5
Synthesis protein	Os03g0769100	0.418	Ribosomal protein s9
Synthesis protein	Os02g0652600	0.459	50s ribosomal protein l19
Synthesis protein	Os03g0704000	0.518	30s ribosomal protein s13
Synthesis protein	Os02g0162500	0.522	Similar to 40s ribosomal protein s14
Synthesis protein	Os03g0315800	0.545	30s ribosomal protein chloroplastic-like
Synthesis protein	Os02g0137200	0.551	50s ribosomal protein l3
Synthesis protein	Os05g0477300	0.590	40s ribosomal protein s26
Synthesis protein	Os08g0559200	0.604	Similar to ribosomal protein s25 (40s ribosomal 25s subunit)
Synthesis protein	Os04g0605900	0.712	60s ribosomal protein l7-1
Synthesis protein	Os02g0591700	0.764	60s ribosomal protein l6
Synthesis protein	Os05g0568300	0.813	Ribosomal protein l12
Synthesis protein	Os03g0751400	0.886	50s ribosomal protein l6
Tca / org	Os08g0191100	-0.401	Similar to iron-responsive element binding protein
Tca / org	Os01g0337900	0.448	Dihydrolipoyl dehydrogenase
Tetrapyrrole synthesis	Os03g0563300	0.514	Magnesium-chelatase subunit chloroplastic-like
Tetrapyrrole synthesis	Os06g0132400	0.555	Magnesium-protoporphyrin ix methyltransferase
Transport	Os09g0250700	-0.520	Uncharacterized protein sll1770-like
Transport	Os01g0127300	0.516	Sufbd family protein
Transport	Os08g0520000	-2.670	Protein brittle- chloroplastic amyloplastic-like

... continuation

Metabolism	Gene-ID	log_2FC	Description
Transport	Os01g0708900	-0.623	Graves disease carrier
Transport	Os01g0871500	-0.598	Oligopeptide transporter
Transport	Os12g0514000	-0.632	Sugar transporter family expressed
Transport	Os01g0279400	-0.623	Major facilitator superfamily antiporter
Transport	Os02g0475400	-0.572	Uncharacterized sodium-dependent transporter yocs-like

3121 **Anexo D – Additional file 4 (Artigo 2)**

3122

Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4337689	4333065	LOC_Os05g03640.1	LOC_Os03g28940.1	0	0.554	0	0.554
4349831	4345848	LOC_Os11g05930.1	LOC_Os08g37456.1	0	0.595	0	0.595
4345848	LOC_Os07g38440.1	LOC_Os08g37456.1	LOC_Os07g38440.1	0	0.402	0	0.402
4348743	4345848	LOC_Os10g30850.1	LOC_Os08g37456.1	0	0.595	0	0.595
4345848	4326612	LOC_Os08g37456.1	LOC_Os01g43090.1	0	0.504	0	0.504
4345848	4330000	LOC_Os08g37456.1	LOC_Os02g40510.1	0	0.753	0	0.753
4345848	4332353	LOC_Os08g37456.1	LOC_Os03g15920.1	0	0.554	0	0.554
4345848	4339778	LOC_Os08g37456.1	LOC_Os05g51150.1	0	0.48	0	0.48
4338354	4334737	LOC_Os05g23740.1	LOC_Os03g62490.1	0	0.52	0	0.52
4338354	4326593	LOC_Os05g23740.1	LOC_Os01g44210.1	0	0.589	0	0.589
4338354	4329960	LOC_Os05g23740.1	LOC_Os02g39870.1	0	0.81	0	0.81
4349426	4338354	LOC_Os10g41710.1	LOC_Os05g23740.1	0	0.568	0	0.568
4338354	OsJ_05302	LOC_Os05g23740.1	LOC_Os02g04460.1	0	0.515	0	0.515
4347356	4338354	LOC_Os09g29840.1	LOC_Os05g23740.1	0	0.63	0	0.63
4351208	4329960	LOC_Os11g47760.1	LOC_Os02g39870.1	0	0.68	0	0.679
4346927	4336056	LOC_Os09g21250.1	LOC_Os04g38750.1	0	0.458	0	0.457
4346927	4332868	LOC_Os09g21250.1	LOC_Os03g24020.1	0	0.4	0	0.4
4346938	4346927	LOC_Os09g21460.1	LOC_Os09g21250.1	0	0.621	0	0.621
4349426	4346927	LOC_Os10g41710.1	LOC_Os09g21250.1	0	0.401	0	0.4
4346927	4344571	LOC_Os09g21250.1	LOC_Os08g03380.1	0	0.438	0	0.438
4346927	4333815	LOC_Os09g21250.1	LOC_Os03g49220.1	0	0.451	0	0.451
4346927	4331442	LOC_Os09g21250.1	LOC_Os03g03020.1	0	0.416	0	0.416
4346927	4331189	LOC_Os09g21250.1	LOC_Os02g57670.1	0	0.403	0	0.403

... continuation

Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4346927	4330740	LOC_Os09g21250.1	LOC_Os02g51480.1	0	0.541	0	0.541
4346927	4330964	LOC_Os09g21250.1	LOC_Os02g54710.1	0	0.421	0	0.42
4346927	4332957	LOC_Os09g21250.1	LOC_Os03g25960.1	0	0.541	0	0.541
4346927	4346032	LOC_Os09g21250.1	LOC_Os08g40430.1	0	0.621	0	0.621
4346927	4331249	LOC_Os09g21250.1	LOC_Os02g58450.1	0	0.586	0	0.586
4346927	4335917	LOC_Os09g21250.1	LOC_Os04g35760.1	0	0.66	0	0.66
4347356	4331058	LOC_Os09g29840.1	LOC_Os02g56040.1	0	0.609	0	0.609
4331058	4329960	LOC_Os02g56040.1	LOC_Os02g39870.1	0	0.459	0	0.459
LOC_Os05g41800.1	4331058	LOC_Os05g41800.1	LOC_Os02g56040.1	0	0.695	0	0.694
4347356	4329960	LOC_Os09g29840.1	LOC_Os02g39870.1	0	0.515	0	0.515
4331058	4329960	LOC_Os02g56040.1	LOC_Os02g39870.1	0	0.459	0	0.459
4349426	4329960	LOC_Os10g41710.1	LOC_Os02g39870.1	0	0.807	0	0.807
4338354	4329960	LOC_Os05g23740.1	LOC_Os02g39870.1	0	0.81	0	0.81
4351208	4329960	LOC_Os11g47760.1	LOC_Os02g39870.1	0	0.68	0	0.679
VIT1.2	4329636	LOC_Os09g23300.1	LOC_Os02g34690.1	0	0.067	0.563	0.574
4351264	4333317	LOC_Os12g01530.1	LOC_Os03g38260.1	0	0.065	0	0.916
4345926	OsJ_09011	LOC_Os08g38720.1	LOC_Os02g58620.1	0	0.079	0.616	0.631
4339898	4337347	LOC_Os06g02220.1	LOC_Os04g57400.1	0	0.113	0.62	0.648
4339898	4331707	LOC_Os06g02220.1	LOC_Os03g06620.1	0	0.142	0.548	0.595
4346803	4333359	LOC_Os09g17740.1	LOC_Os03g39610.1	0.981	0.749	0	0.748
4333359	OsJ_01305	LOC_Os03g39610.1	LOC_Os01g17170.1	0	0.487	0	0.487
4333359	4329439	LOC_Os03g39610.1	LOC_Os02g30320.1	0	0.475	0	0.475
4341930	4333359	LOC_Os06g47970.1	LOC_Os03g39610.1	0	0.465	0	0.465
4344439	4333359	LOC_Os08g01380.1	LOC_Os03g39610.1	0	0.538	0	0.538
4333359	4324599	LOC_Os03g39610.1	LOC_Os01g41710.1	0.982	0.991	0	0.99
4346803	4333359	LOC_Os09g17740.1	LOC_Os03g39610.1	0.981	0.749	0	0.748

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Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4344439	4324599	LOC_Os08g01380.1	LOC_Os01g41710.1	0	0.485	0	0.485
4333359	4324599	LOC_Os03g39610.1	LOC_Os01g41710.1	0.982	0.991	0	0.99
4345971	4332745	LOC_Os08g39430.1	LOC_Os03g21560.1	0	0.547	0	0.547
4345971	4333370	LOC_Os08g39430.1	LOC_Os03g39830.1	0	0.464	0	0.463
4337500	4326537	LOC_Os04g59440.1	LOC_Os01g71190.1	0	0.4	0	0.4
4328558	4326537	LOC_Os02g09590.1	LOC_Os01g71190.1	0	0.526	0	0.526
4331442	4326537	LOC_Os03g03020.1	LOC_Os01g71190.1	0	0.483	0	0.484
4340015	4326537	LOC_Os06g04150.1	LOC_Os01g71190.1	0	0.49	0	0.491
4351015	4326537	LOC_Os11g42490.1	LOC_Os01g71190.1	0	0.481	0	0.481
4326537	4327541	LOC_Os01g71190.1	LOC_Os01g59060.1	0	0.429	0	0.429
4337500	4326537	LOC_Os04g59440.1	LOC_Os01g71190.1	0	0.4	0	0.4
4339170	4326537	LOC_Os05g41190.1	LOC_Os01g71190.1	0	0.607	0	0.607
4343402	4326537	LOC_Os07g33660.1	LOC_Os01g71190.1	0	0.424	0	0.424
LOC_Os02g03330.1	4326537	LOC_Os02g03330.1	LOC_Os01g71190.1	0	0.407	0	0.408
4341612	4326537	LOC_Os06g43140.1	LOC_Os01g71190.1	0	0.582	0	0.582
4340929	4326537	LOC_Os06g22690.1	LOC_Os01g71190.1	0	0.409	0	0.409
4329037	4326537	LOC_Os02g18450.1	LOC_Os01g71190.1	0	0.916	0	0.916
4329888	4326537	LOC_Os02g38820.1	LOC_Os01g71190.1	0	0.527	0	0.527
4326537	OsJ_01305	LOC_Os01g71190.1	LOC_Os01g17170.1	0	0.6	0	0.6
4334555	4326537	LOC_Os03g60100.1	LOC_Os01g71190.1	0	0.424	0	0.424
LOC_Os03g52660.1	4326537	LOC_Os03g52660.1	LOC_Os01g71190.1	0	0.415	0	0.415
4332466	4326537	LOC_Os03g17580.1	LOC_Os01g71190.1	0	0.453	0	0.453
4350622	4326537	LOC_Os11g32320.1	LOC_Os01g71190.1	0	0.533	0	0.533
4332652	4326537	LOC_Os03g20100.1	LOC_Os01g71190.1	0	0.642	0	0.642
4344439	4326537	LOC_Os08g01380.1	LOC_Os01g71190.1	0	0.664	0	0.664
4345686	4326537	LOC_Os08g34220.1	LOC_Os01g71190.1	0	0.838	0	0.838

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Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4333370	4326537	LOC_Os03g39830.1	LOC_Os01g71190.1	0	0.822	0	0.822
4342069	4326537	LOC_Os06g50130.1	LOC_Os01g71190.1	0	0.476	0	0.476
4338460	4326537	LOC_Os05g27100.1	LOC_Os01g71190.1	0	0.569	0	0.569
4343582	4326537	LOC_Os07g37230.1	LOC_Os01g71190.1	0	0.462	0	0.461
4326537	OsJ_09047	LOC_Os01g71190.1	13114.m00116	0	0.442	0	0.442
4331189	4326537	LOC_Os02g57670.1	LOC_Os01g71190.1	0	0.478	0	0.478
4334247	4326537	LOC_Os03g55930.1	LOC_Os01g71190.1	0	0.482	0	0.482
4326537	4326593	LOC_Os01g71190.1	LOC_Os01g44210.1	0	0.447	0	0.447
4333370	4332745	LOC_Os03g39830.1	LOC_Os03g21560.1	0	0.682	0	0.682
4339170	4332745	LOC_Os05g41190.1	LOC_Os03g21560.1	0	0.508	0	0.508
4344439	4332745	LOC_Os08g01380.1	LOC_Os03g21560.1	0	0.577	0	0.577
4345971	4332745	LOC_Os08g39430.1	LOC_Os03g21560.1	0	0.547	0	0.547
4342069	4332745	LOC_Os06g50130.1	LOC_Os03g21560.1	0	0.664	0	0.664
4340015	4332745	LOC_Os06g04150.1	LOC_Os03g21560.1	0	0.626	0	0.626
4336886	4332745	LOC_Os04g51300.1	LOC_Os03g21560.1	0	0.655	0	0.655
4352215	4333370	LOC_Os12g29570.1	LOC_Os03g39830.1	0	0.429	0	0.429
4343634	4332868	LOC_Os07g38000.1	LOC_Os03g24020.1	0	0.4	0	0.4
4343634	4338460	LOC_Os07g38000.1	LOC_Os05g27100.1	0	0.487	0	0.487
4343634	4327541	LOC_Os07g38000.1	LOC_Os01g59060.1	0	0.46	0	0.46
4343634	4331189	LOC_Os07g38000.1	LOC_Os02g57670.1	0	0.447	0	0.447
4343634	4341869	LOC_Os07g38000.1	LOC_Os06g46930.1	0	0.44	0	0.44
4343634	OsJ_07762	LOC_Os07g38000.1	LOC_Os02g43600.1	0	0.652	0	0.652
4343634	4331442	LOC_Os07g38000.1	LOC_Os03g03020.1	0	0.525	0	0.525
4343634	4340015	LOC_Os07g38000.1	LOC_Os06g04150.1	0	0.425	0	0.424
4343634	4334247	LOC_Os07g38000.1	LOC_Os03g55930.1	0	0.507	0	0.507
4343634	OsJ_22993	LOC_Os07g38000.1	LOC_Os07g04160.1	0	0.446	0	0.446

... continuation

Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4344439	4326593	LOC_Os08g01380.1	LOC_Os01g44210.1	0	0.565	0	0.565
4344439	4338460	LOC_Os08g01380.1	LOC_Os05g27100.1	0	0.405	0	0.405
4344439	OsJ_01305	LOC_Os08g01380.1	LOC_Os01g17170.1	0	0.783	0	0.783
4344439	4334247	LOC_Os08g01380.1	LOC_Os03g55930.1	0	0.435	0	0.435
4344439	4332652	LOC_Os08g01380.1	LOC_Os03g20100.1	0	0.486	0	0.486
4344439	4333370	LOC_Os08g01380.1	LOC_Os03g39830.1	0	0.797	0	0.797
4344439	4332745	LOC_Os08g01380.1	LOC_Os03g21560.1	0	0.577	0	0.577
4344439	4329037	LOC_Os08g01380.1	LOC_Os02g18450.1	0	0.425	0	0.425
4344439	4340015	LOC_Os08g01380.1	LOC_Os06g04150.1	0	0.46	0	0.46
4344439	LOC_Os03g52660.1	LOC_Os08g01380.1	LOC_Os03g52660.1	0	0.502	0	0.502
4344439	4326537	LOC_Os08g01380.1	LOC_Os01g71190.1	0	0.664	0	0.664
4344439	4329888	LOC_Os08g01380.1	LOC_Os02g38820.1	0	0.48	0	0.48
4344439	4324599	LOC_Os08g01380.1	LOC_Os01g41710.1	0	0.485	0	0.485
4344439	4342069	LOC_Os08g01380.1	LOC_Os06g50130.1	0	0.568	0	0.568
4344439	OsJ_05302	LOC_Os08g01380.1	LOC_Os02g04460.1	0	0.463	0	0.463
4344439	4333359	LOC_Os08g01380.1	LOC_Os03g39610.1	0	0.538	0	0.538
4344439	4339170	LOC_Os08g01380.1	LOC_Os05g41190.1	0	0.815	0	0.815
4344439	4328558	LOC_Os08g01380.1	LOC_Os02g09590.1	0	0.684	0	0.684
4344439	4334555	LOC_Os08g01380.1	LOC_Os03g60100.1	0	0.469	0	0.469
4344439	4343219	LOC_Os08g01380.1	LOC_Os07g29410.1	0	0.419	0	0.419
4340015	4336886	LOC_Os06g04150.1	LOC_Os04g51300.1	0	0.559	0	0.559
4345686	4336886	LOC_Os08g34220.1	LOC_Os04g51300.1	0	0.583	0	0.583
4336886	4331189	LOC_Os04g51300.1	LOC_Os02g57670.1	0	0.55	0	0.551
4339170	4336886	LOC_Os05g41190.1	LOC_Os04g51300.1	0	0.549	0	0.549
4336886	4334555	LOC_Os04g51300.1	LOC_Os03g60100.1	0	0.527	0	0.527
4336886	4332745	LOC_Os04g51300.1	LOC_Os03g21560.1	0	0.655	0	0.655

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Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4336886	4333233	LOC_Os04g51300.1	LOC_Os03g34040.1	0	0.466	0	0.465
4336886	4331442	LOC_Os04g51300.1	LOC_Os03g03020.1	0	0.507	0	0.507
4336886	OsJ_01975	LOC_Os04g51300.1	LOC_Os01g32830.1	0	0.428	0	0.428
4336886	4333370	LOC_Os04g51300.1	LOC_Os03g39830.1	0	0.411	0	0.411
4336886	OsJ_07762	LOC_Os04g51300.1	LOC_Os02g43600.1	0	0.632	0	0.632
4343402	4328586	LOC_Os07g33660.1	LOC_Os02g09940.1	0	0.405	0	0.405
4332868	4328586	LOC_Os03g24020.1	LOC_Os02g09940.1	0	0.419	0	0.418
OsJ_07762	4328586	LOC_Os02g43600.1	LOC_Os02g09940.1	0	0.508	0	0.508
4340015	4328586	LOC_Os06g04150.1	LOC_Os02g09940.1	0	0.65	0	0.65
4338460	4328586	LOC_Os05g27100.1	LOC_Os02g09940.1	0	0.603	0	0.603
4328586	OsJ_01975	LOC_Os02g09940.1	LOC_Os01g32830.1	0	0.431	0	0.431
4336056	4328586	LOC_Os04g38750.1	LOC_Os02g09940.1	0	0.435	0	0.435
4352413	4328586	LOC_Os12g34890.1	LOC_Os02g09940.1	0	0.5	0	0.5
4331442	4328586	LOC_Os03g03020.1	LOC_Os02g09940.1	0	0.559	0	0.559
4332652	4328586	LOC_Os03g20100.1	LOC_Os02g09940.1	0	0.42	0	0.42
4345686	4328586	LOC_Os08g34220.1	LOC_Os02g09940.1	0	0.454	0	0.454
4334247	4328586	LOC_Os03g55930.1	LOC_Os02g09940.1	0	0.612	0	0.612
4334555	4328586	LOC_Os03g60100.1	LOC_Os02g09940.1	0	0.463	0	0.463
4340929	4328586	LOC_Os06g22690.1	LOC_Os02g09940.1	0	0.517	0	0.517
4334131	4328586	LOC_Os03g54040.1	LOC_Os02g09940.1	0	0.438	0	0.438
4331189	4328586	LOC_Os02g57670.1	LOC_Os02g09940.1	0	0.523	0	0.523
4346329	4331442	LOC_Os08g44770.1	LOC_Os03g03020.1	0	0.658	0	0.658
4346329	4333233	LOC_Os08g44770.1	LOC_Os03g34040.1	0	0.451	0	0.451
4346329	4334247	LOC_Os08g44770.1	LOC_Os03g55930.1	0	0.446	0	0.446
4343987	4333342	LOC_Os07g43570.1	LOC_Os03g38950.1	0	0.468	0	0.468
4343987	4331358	LOC_Os07g43570.1	LOC_Os03g02020.2	0	0.492	0	0.491

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Node1	Node2	Node1_external_id	Node2_external_id	Homology	Coexpression	Textmining	Combined_score
4343987	4339778	LOC_Os07g43570.1	LOC_Os05g51150.1	0	0.73	0	0.73
4343987	4343219	LOC_Os07g43570.1	LOC_Os07g29410.1	0	0.437	0	0.436
4345611	4343987	LOC_Os08g32930.1	LOC_Os07g43570.1	0	0.57	0	0.57
4347775	4343987	LOC_Os09g37620.1	LOC_Os07g43570.1	0	0.518	0	0.518
4343987	4343986	LOC_Os07g43570.1	LOC_Os07g43560.1	0.943	0.992	0	0.992
4343987	4326612	LOC_Os07g43570.1	LOC_Os01g43090.1	0	0.409	0	0.408
4343987	4341038	LOC_Os07g43570.1	LOC_Os06g28194.1	0	0.628	0	0.627
4343987	4330451	LOC_Os07g43570.1	LOC_Os02g47510.1	0	0.758	0	0.758
4349671	4343987	LOC_Os11g03580.1	LOC_Os07g43570.1	0	0.464	0	0.463
4343987	4343652	LOC_Os07g43570.1	LOC_Os07g38230.1	0	0.597	0	0.597
OsJ_34121	4343987	LOC_Os11g32880.1	LOC_Os07g43570.1	0	0.428	0	0.428
4347775	4343986	LOC_Os09g37620.1	LOC_Os07g43560.1	0	0.42	0	0.42
4343986	4339778	LOC_Os07g43560.1	LOC_Os05g51150.1	0	0.41	0	0.409
4343986	4343219	LOC_Os07g43560.1	LOC_Os07g29410.1	0	0.423	0	0.423
4343987	4343986	LOC_Os07g43570.1	LOC_Os07g43560.1	0.943	0.992	0	0.992
4343986	4343652	LOC_Os07g43560.1	LOC_Os07g38230.1	0	0.634	0	0.634

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