RÔMULO FREDSON DUARTE

BIOFORTIFICAÇÃO DE ARROZ COM ZINCO:
QUANTIFICAÇÃO E EXPRESSÃO GÊNICA

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ciência do Solo, área de concentração em Fertilidade do Solo e Nutrição de Plantas, para obtenção do titulo de Doutor.

Orientador

Dr. Valdemar Faquin

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APROVADA em 20 de maio de 2015.

Dr. Luiz Roberto Guimarães Guilherme	UFLA
Drª. Joelma Pereira	UFLA
Dr. Luciano Vilela Paiva	UFLA
Drª. Chanakan Prom-u-thai	CMU - TAILÂNDIA
Dr. Milton Ferreira de Moraes	UFMT

Dr. Valdemar Faquin

Orientador

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RESUMO GERAL

Zinco (Zn) é um dos micronutrientes mais importantes para os seres humanos, plantas e animais. No entanto, o consumo de alimentos pobre em Zn, como é o caso de dietas ricas em arroz, pode levar à desnutrição com Zn nas pessoas. Por isso, o uso da biofortificação através do aumento do teor de Zn nos grãos, por exemplo, tem sido uma das abordagens mais sustentáveis para atenuar a deficiência de Zn. Assim, os objetivos desse trabalho por meio de dois experimentos foram avaliar: a) a variação da concentração de Zn nos grãos de acessos de arroz de terras altas do Brasil por meio da determinação por digestão ácida seguida de análise via espectrofotômetro de absorção atômica e pelo método de coloração por dithizone (DTZ); b) a intensidade de coloração de Zn nos grãos de arroz através do programa ImageJ software; c) o envolvimento de genes relacionados à absorção e translocação de Zn pela análise de expressão gênica durante diferentes estágios da planta de arroz; e, d) a relativa contribuição da absorção e translocação de Zn da planta para os grãos usando genótipos de arroz com diferentes concentrações de Zn em suas sementes. A partir dos resultados do primeiro experimento concluiu-se que o uso do método de coloração por DTZ, em conjunto com o ImageJ software, proporcionaram a identificação da variação da concentração de Zn nos grãos e entre as partes dos grãos (embrião, endosperma e aleurona) entre os genótipos de arroz estudados. Essa é uma descoberta importante àqueles interessados em desenvolver genótipos de arroz com alto teor de Zn nos grãos visando atenuar o problema de deficiência de Zn na população mundial. Assim, sugere-se essa metodologia para uma triagem rápida, quando se tem como objetivo o desenvolvimento de germoplasmas de arroz potenciais em acumular Zn. No entanto, seria interessante testar essa metodologia em maiores lotes de genótipos de arroz visando refinar esta metodologia. O segundo experimento observou que os genótipos de alta concentração de Zn na semente não foram os mais eficientes em Zn, porém foram encontrados os maiores teores de Zn nos grãos, o que pode estar associado à translocação de Zn da raiz para a parte aérea. Análises de expressão por sqPCR identificaram principalmente o *Os*ZIP4 como o gene mais expresso, independentemente das fases de crescimento das plantas. Ademais, análises por qPCR também mostraram diferenças nos níveis de expressão nos diferentes tecidos das plantas cultivadas sob tratamentos com Zn. No entanto, outras investigações utilizando um grande conjunto de genótipos representativos para estudos com a biofortificação, bem como identificar características fisiológicas favoráveis ao acúmulo de Zn nos grãos podem ser necessárias.

Palavras-chave: *Oryza sativa*. Zinco. Biofortificação. Deficiência de Zn. Coloração com Zn. Expressão gênica.

GENERAL ABSTRACT

Zinc (Zn) is one of the most important micronutrients for humans, plants and animals. However, the consumption of food with low Zn concentration (e.g., rice cereal diets) can lead to human zinc deficiency. Thus, the use of the biofortification through the increasing of the grain zinc content, for instance, has been one of the most sustainable approaches to alleviate the Zn deficiency. In this study two experiments were conducted where we evaluated: a) the variation of Zn concentration in rice grain among accessions from Brazil through the determination by acid digestion followed by analyzes using atomic absorption spectrophotometer and dithizone staining method (DTZ); b) the Zn staining intensity in the rice grains by using the ImageJ software analysis; c) the genes involved in metal homeostasis through the gene expression analysis during different rice plant stages; d) the relative contribution of uptake and translocation of Zn from plant to grains using rice genotypes with different seed Zn concentration. The results of first experiment showed that using the DTZ staining method together with ImageJ software analysis led to the identification of the variation of Zn concentration in grains and between grains tissues (embryo, endosperm and aleurone layer) among the rice genotypes studied. This is an important finding for rice breeders interested in developing high-density Zn genotypes to solve the Zn deficiency problem among the world's population. Thus, we suggest this methodology as a rapid screening procedure for the development of potential rice germplasm in accumulating Zn. However, it would be interesting to test this methodology in a large set of rice genotypes aiming for further refined calibration. In the second experiment observed that the high density Zn genotypes are not the most Zn efficient genotypes, but they promoted higher grain Zn content, which can be associated to root-shoot Zn translocation. Analysis of gene expression by sqPCR identified *Os*ZIP4 as the more expressed gene independently of the plant growth stages. Additionally, qPCR analysis showed also differences in relative expression levels in different plant tissues grown under Zn treatments. However, further investigations following a wide group of representative genotypes for studies with biofortification, and identify physiological characteristics favorable to Zn accumulation in the grain may be necessary.

Keywords: *Oryza sativa*. Zinc. Biofortification. Zn deficiency. Zn staining. Gene expression.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

Segundo projeções recentes da Divisão de População do Departamento de Economia e Assuntos Sociais das Nações Unidas, haverá um incremento na população mundial passando de cerca de 7,2 bilhões alcançados em 2013 para mais de 10.9 bilhões de pessoas no final desse século (UNITED NATIONS, DEPARTMENT OF ECONOMIC AND SOCIAL AFFAIRS, 2012). Desse total, cerca de 805 milhões de pessoas sofrem subnutrição crônica e 1,4 bilhão se encontram em extrema pobreza e, por conta disso, muitos países ainda não atingiram o primeiro Objetivo de Desenvolvimento do Milênio (The Millennium Development Goals - MDGs) o qual propõe a redução pela metade, entre 1990 e 2015, da proporção de pessoas que vivem na fome e extrema pobreza (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2014). Na verdade, durante esse período, os números apontam para uma redução na desnutrição humana na ordem de 18,7% para 11,3% a nível mundial e de 23,4% para 13,5% para países em desenvolvimento. Dados da Organização Mundial de Saúde (World Health Organization) revelam que mais de 2 bilhões de pessoas no mundo são atingidos por deficiências vitamínicas e minerais, tais como iodo, ferro, vitamina A e zinco, com consequências importantes para a saúde. Tal diagnóstico ainda aborda que existe uma situação mais grave em mulheres e crianças, principalmente aquelas que vivem em países em desenvolvimento (KENNEDY; NANTEL; SHETTY, 2003; UNITED NATIONS, DEPARTMENT OF ECONOMIC AND SOCIAL AFFAIRS, 2012; UNITED NATIONS SYSTEM STANDING COMMITTEE ON NUTRITION, 2004; WORLD HEALTH ORGANIZATION, 2000).

O arroz é o alimento básico consumido por mais da metade da população mundial e o Brasil é o país não asiático de maior produção e consumo de seus grãos. Entretanto, em países em desenvolvimento, esse alimento fornece apenas 27% de energia, 20% de proteína e 3% de gordura na dieta (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2004a; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 2008; REDOÑA, 2004; SPEROTTO et al., 2009), valores considerados baixos. Além do mais, o arroz é uma fonte pobre em micronutrientes, tais como Fe e Zn, o que torna a busca por variedades de arroz com boa qualidade nutricional cada vez mais importante (DUAN; SUN, 2005). Assim, além de desenvolver investigações quanto ao potencial de cultivares de arroz em acumular Zn e outros micronutrientes, estudos da localização e dinâmica desse elemento na planta e na semente fazem parte de uma estratégia interessante para auxiliar em pesquisas futuras de biofortificação de alimentos. Segundo Ozturk et al. (2006), informações sobre a dinâmica de acumulação de Zn em sementes poderia ser útil, por exemplo, na determinação do melhor momento para aplicações foliares de Zn. Dessa forma, tais medidas auxiliariam o produtor ou pesquisador a tomar medidas mais pontuais visando à busca por genótipos mais eficientes em acumular mais Zn.

A biofortificação é uma prática que visa o desenvolvimento de culturas básicas com maior teor de micronutrientes por meio da adoção de melhores práticas tradicionais de cultivo e da biologia molecular moderna (NESTEL et al., 2006). Sendo assim, o objetivo maior é melhorar a qualidade nutricional do alimento sem causar danos ou riscos à saúde humana. Dessa forma, as últimas pesquisas no mundo inteiro têm focado em estratégias de melhoramento vegetal por meio da biofortificação de alimentos em culturas básicas da alimentação humana, a fim de reduzir a desnutrição mundial relacionada a micronutrientes

(BOUIS; WELCH, 2010; DUARTE et al., 2013; RAMOS et al., 2010; REIS et al., 2014; SAUTTER et al., 2006; WHITE; BROADLEY, 2005).

Do ponto de vista da biologia molecular, a biofortificação faz uso do melhoramento de plantas e, ou, abordagens transgênicas para desenvolver novos genótipos com potencial para aumentar a concentração de nutrientes em partes comestíveis das plantas (WHITE; BROADLEY, 2005). Assim, trabalhos apontam por mecanismos moleculares que regulam a mobilização do elemento (Fe e Zn, por exemplo) das folhas para as sementes em desenvolvimento, como tem sido observado na cultura do arroz (SPEROTTO et al., 2009; SPEROTTO et al., 2010). Nessa perspectiva, Banerjee e Chandel (2011) ao estudarem 25 genes como candidatos prováveis em expressar a absorção, transporte e redistribuição de Fe e Zn em cultivo de arroz, encontraram expressão diferenciada desses genes nos tecidos foliares, radicular e na folha bandeira. Já Sperotto et al. (2010) observaram que o nível de expressão de 9 dos 25 genes estudados (*Os*YSL6, *Os*YSL8, *Os*YSL14, *Os*NRAMP1, *Os*NRAMP7, *Os*NRAMP8, *Os*NAS1, *Os*FRO1 e *Os*NAC5) na folha bandeira se correlacionou significativamente com os teores de Fe, e, ou, Zn nas sementes, o que sugere genes alvos para manipulação desses micronutrientes em grãos de arroz. Apesar da existência de muitos trabalhos se envolvam a questão de quais genes são expressos durante o desenvolvimento reprodutivo das plantas, poucos são aqueles que trazem resultados conclusivos.

Sendo assim, a agricultura recente deverá postular novas políticas baseadas na produção de alimentos que não apenas forneçam calorias suficientes para suprir as necessidades de energia da população, mas também ofereçam todos os nutrientes essenciais para uma adequada nutrição humana (BOUIS; WELCH, 2010). Para isso, as investigações de processos envolvidos no enchimento de grãos bem como o entendimento de como estes processos ocorrem e quais são os genes responsáveis pelo transporte de nutrientes, são de

extrema importância para programas de melhoramento convencional e com uso de engenharia genética. Além do mais, tais ferramentas serão abordagens promissoras para o aumento dos teores de nutrientes, incluindo o Zn, em alimentos de necessidade básica (staple foods) e culturas em programas de biofortificação.

Nesse sentido, esta pesquisa foi realizada com os objetivos de: (i) avaliar os genótipos de arroz de terras altas potenciais em acumular Zn por meio do método de coloração do grão com dithizone (DTZ) analisado pelo software ImajeJ; e, (ii) avaliar a expressão gênica de genes relacionados com o transporte e translocação de zinco na planta usando genótipos de arroz com diferentes concentrações de Zn em seus grãos.

2 REFERENCIAL TEÓRICO

A crescente demanda por alimentos com melhor qualidade (alimentos biofortificados), aqueles baseados em maiores teores de nutrientes essenciais à nutrição humana, especialmente zinco (Zn), ferro (Fe), iodo (I), selênio (Se) e vitaminas, está cada vez mais em evidência no mundo. Por outro lado, à medida que a produção de alimentos na agricultura avança, ou seja, quanto se busca produzir cada vez mais, a qualidade fica aquém daquela exigida pela populção devido ao próprio “efeito da diluição” dos nutrientes aplicados ou a certa variabilidade genética das espécies cultivadas. Em decorrência disso, adotar estratégias que relacionam a produção de alimentos com aspectos de qualidade, e.g., maiores teores de nutrientes e vitaminas, são extremamente importantes.

A biofortificação de alimentos surge como uma das importantes ferramentas para combate às deficiências em micronutrientes e vitaminas. Ela complementará as intervenções em nutrição existentes, além de proporcionar

uma maneira sustentável e de baixo custo para alcançar as populações com limitado acesso aos sistemas formais de mercado e de saúde.

2.1 A desnutrição humana

A desnutrição é a condição que se desenvolve quando o corpo não recebe uma quantidade desejada de vitaminas, minerais e outros nutrientes de que necessita para manter os tecidos saudáveis e funcionamento dos órgãos. Mundialmente, é o fator de risco mais importante para a causa de doenças e mortes nos países em desenvolvimento, sendo centenas de milhões de mulheres grávidas e crianças pequenas particularmente as mais afetadas (MURRAY; LOPEZ, 1997). Estudos mostram que cerca de 53% das mortes entre crianças menores de 5 anos de idade estão associadas à desnutrição (BLACK et al., 2003; FOOD AND AGRICULTURAL ORGANIZATION, 2004b; MÜLLER; KRAWINKEL, 2004b; MÜLLER; KRAWINKEL, 2005; MÜLLER et al., 2003; NEMER; GELBAND; JHA, 2001). Entre as regiões mais atingidas estão a África Subsaariana, a Ásia Meridional, o Sudeste Asiático, a América Latina e o Caribe, por consumirem dietas pobres em micronutrientes essenciais, particularmente ferro, vitamina A, iodo e zinco (WORLD HEALTH ORGANIZATION, 2013).

Entre as carências nutricionais, a deficiência de zinco em humanos, recentemente, é uma das que vem ganhando mais atenção, uma vez que ela é bastante comum em todo o mundo. Dados estimam que a deficiência de zinco afeta em média um terço da população mundial, além de contribuir para a morte de mais de 450.000 crianças menores que cinco anos anualmente (ALLOWAY, 2008; BLACK et al., 2008). Últimas análises no Consenso de Copenhague revelaram a deficiência de Zn, juntamente com a deficiência de vitamina A, como questões de prioridade mundial e concluíram que a eliminação do

problema com deficiência de Zn resultará em altos impactos imediatos e alto retorno para humanidade no mundo em desenvolvimento (ALLOWAY, 2008). Entre outras deficiências a nível de micronutrientes, dados ainda estimam que existam cerca de 4 a 5 bilhões de pessoas com deficiência de Fe, onde 37% são anêmicas, sendo considerado o distúrbio nutritrional mais comum no mundo; 0,5 a 1,0 milhão com deficiência de Se e acima de 800 milhões de pessoas com deficiência de iodo (ALLEN et. al., 2006; COMBS JUNIOR, 2001; HOTZ; BROWN, 2004; WELCH, 2008).

2.2 O Zinco nas plantas

O zinco (Zn) é um micronutriente essencial nos processos biológicos de todos seres humanos, animais e plantas o qual desempenha um papel fundamental para o funcionamento de mais de 300 enzimas em várias espécies. Enzimas que contém zinco como constituinte estrutural exercem uma participação essencial no metabolismo dos carboidratos, lipídeos, proteínas e ácidos nucleicos (LUKASKI, 1995; VALLEE; FALCHUK, 1993). Além disso, em plantas superiores, o Zn modula a atividade de diversos tipos de enzimas tais como desidrogenases, aldolases, isomerases, transfosforilases, além de RNA e DNA polimerases (MARSCHNER, 2012).

Estima-se que na maioria da parte aérea de uma planta não acumuladora, sob condições normais de Zn, os teores desse elemento adequados ao seu bom crescimento e desenvolvimento estão entre de 30 e 100 μg g^{-1} de matéria seca, sendo que os valores acima de 300 μg g^{-1} são geralmente considerados tóxicos (MARSCHNER, 1995). A ocorrência de Zn nas plantas se dá através de complexos de baixo peso molecular, metaloproteínas de armazenagem, íons lívres e formas insolúveis associadas às paredes celulares (ALLOWAY, 2008).

Dentre esses, os complexos de baixo peso molecular são normalmente as formas mais abundantes de Zn e são provavelmente a forma mais ativa do metal (BROWN; CAKMAK; ZHANG, 1993). A presença do Zn nas plantas ainda depende de sua concentração no solo e biodisponibilidade, a qual é modulada por vários fatores físicos e químicos do solo. Além do mais, a solubilidade do Zn no solo diminui devido ao elevado nível de carbonato de cálcio, presença de óxidos, pH e níveis baixos de matéria orgânica e umidade do solo (CAKMAK, 2011; ROBSON, 1994).

Em plantas, bem como outros organismos, a manutenção da concentração de metais essenciais dentro dos limites fisiológicos e a capacidade de minimizar o efeito dos elementos tóxicos ocorrem devido à presença de uma rede de mecanismos homeostáticos que servem para controlar a absorção, acumulação, transporte e desintoxicação de metais (CLEMENS, 2001). Um fato importante é que, no sentido de entender a dinâmica do Zn bem como a de outros metais em diversas partes e estágios da planta de arroz, vários trabalhos apontam sobre a homeostase de alguns elementos, os quais são estritamente regulados por transportadores específicos associados ao influxo, efluxo e compartimentalização intracelular, bem como outros mecanismos envolvidos, porém ainda desconhecidos (BASHIR; ISHIMARU; NISHIZAWA, 2012; ISHIMARU et al., 2005; SPEROTTO et al., 2009; SPEROTTO et al., 2010). Dessa forma, conhecer esses mecanismos envolvidos na absorção, transporte e translocação de Zn são essenciais para futuros trabalhos de biofortificação.

2.3 A importância do zinco na saúde humana

A alta incidência de solos com deficiência de Zn (Figura 1), tido como problema global, associado com a ingestão de alimentos à base de cereais com baixos teores e biodisponibilidade desse elemento parece estar entre as

principais causas de deficiência de Zn na população mundial (CAKMAK, 2008).

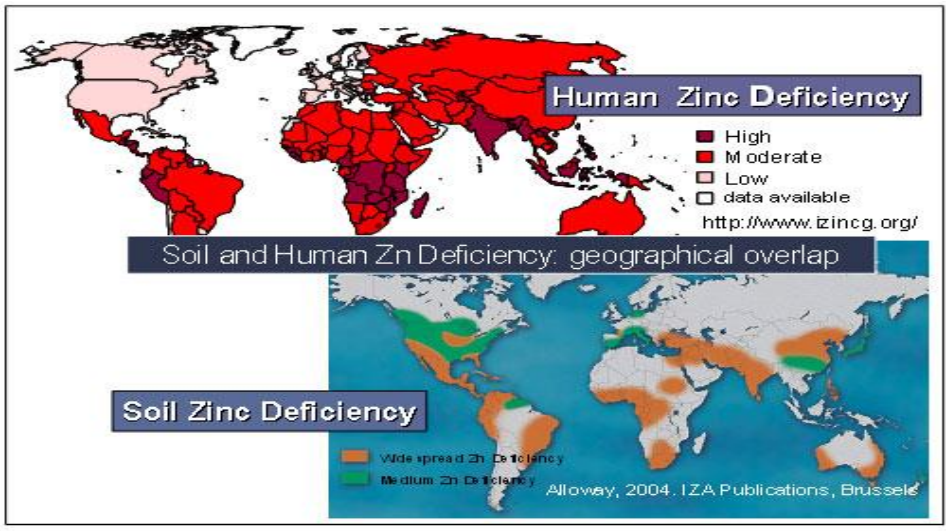

Figura 1 Distribuição das áreas afetadas por deficiência de Zn
Fonte: Alloway (2004)

Regiões onde ocorre deficiência de Zn nos solos resultam em baixos teores de Zn nas plantas e, consequentemente, ocorrência de deficiência generalizada de Zn na população. Dessa forma, grandes problemas têm ocorrido em países como a Índia, China, Paquistão, Irã e Turquia, onde os solos possuem baixa disponibilidade de Zn e cultivam cereais, principais fontes de calorias diariamente (ALLOWAY 2004; CAKMAK et al., 1999; HOTZ; BROWN, 2004). No Brasil, a deficiência de zinco é a mais comum entre os micronutrientes, principalmente em solos sob o cerrado e solos arenosos. Tal cenário contribui para a diminuição da qualidade nutricional dos grãos, além de causar problemas de saúde nos seres humanos, principalmente onde os cereais (arroz, trigo e milho) são as principais fontes para a alimentação (FRAIGE; CRESPILHO; REZENDE, 2007; GRAHAM; WELCH, 1996; KALAYCI et al., 1999).

Quanto ao Zn como contribuinte à saúde humana, sabe-se que esse elemento desempenha funções chaves e fundamentais. Assim, o Zn está relacionado ao crescimento e desenvolvimento físico, funcionamento do sistema imunológico, associado à saúde reprodutiva, função sensorial e desenvolvimento neurocomportamental (HOTZ; BROW, 2004). Doses diárias recomendadas de Zn, as quais são baseadas em função da idade, gênero, tipo de dieta e outros fatores, variam em torno de 3 a 17 mg de Zn por dia (HOTZ; BROW, 2004; SHARMA et al., 2013).

2.4 Genes envolvidos no transporte do zinco na planta

Estudos prévios têm demonstrado que a manutenção da homeostase de íons em toda a planta depende de uma série de transportadores, incluindo os membros das famílias ZRT (Zinc-regulated transporter), IRT (iron-regulated transporter), ZIP (Zinc-regulated transporters, Iron-regulated transporter-like Protein) e Nramp (Natural resistance-associated macrophage Protein), dentre outros (COLANGELO; GUERINOT, 2006). A homeostase é um processo importante para manter uma adequada concentração do elemento na célula, caso do zinco e ferro, sendo imprescindíveis para um ideal crescimento e produção das plantas.

Os transportadores ZIP, apontados como um dos mais estudados, atuam na homeostase através do transporte de cátions para o citoplasma. Análises mostram a capacidade desses transportadores em moverem uma variedade de cátions bivalentes, incluindo Fe^{2+}, Zn^{2+}, Mn^{2+} e Cd^{2+} (COLANGELO; GUERINOT, 2006; GROTZ; GUERINOT, 2006). Na verdade, cada gene possui maior ou menor especificidade para desempenhar uma certa função em um organismo. Na planta, transcrições dos genes *Os*ZIP1 e *Os*ZIP4 foram confirmados sob condições de deficiência de Zn, Fe, Mn e Cu, enquanto a

expressão de *Os*ZIP4 foi altamente induzida pela deficiência de Zn em várias partes da planta de arroz (ISHIMARU et al., 2005). Os genes ZIP1 e ZIP3 parece ser específicos para raiz, enquanto transportadores ZIP4 tem acumulado tanto nas raízes quanto na parte aérea das plantas cultivadas sob condições de deficiência de Zn (GROTZ; GUERINOT, 2006; ISHIMARU et al., 2005). Na cultura do arroz, uma das culturas mais importantes para a alimentação humana, também várias proteínas ZIP tem sido caracterizadas (ISHIMARU et al., 2005; ISHIMARU et al., 2006; RAMESH et al., 2003).

Outro fato importante a ser considerado na regulação da absorção e transporte do metal, se refere ao recurso de algumas plantas em produzir ou secretar ácidos muginéicos (MAs) da família dos fitosideróforos (PS), os quais são muito comuns em gramíneas sob condição de deficiência do metal e, estão envolvidos na aquisição de ferro (Fe) do solo. Assim, vários MAs tem a capacidade de formar quelatos com cátions divalentes, desempenhando um papel importante na translocação de Zn nas plantas. Nicotianamina (NA), precursor biossintético de MA, é propício a ser um dos quelantes principais de metais, incluindo Fe e Zn, em todas as plantas superiores. Além do papel central da NA, MA em gramíneas também parece estar envolvidos na translocação de Fe e Zn (ISHIMARU et al., 2005; LEE et al., 2012; SINCLAIR; KRÄMER, 2012; SUZUKI et al., 2006).

Portanto, diante da importância dos metais para a sobrevivência, exercendo função apropriada às plantas e demais organismos, além da importância das plantas como fontes de energia, nutrientes e vitaminas, é notório a busca cada vez mais por pesquisas que contemplem essa temática, sobretudo na associação da biologia molecular com a produção de alimentos. Como resultado disso, futuros estudos de melhoramento convencional ou biotecnológico devem ser gerados e priorizados.

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SEGUNDA PARTE - ARTIGOS

ARTIGO 1

Determination of Zinc in Rice Grains by DTZ Staining Technique and ImageJ Software Analysis

(Normas de publicação da revista *Jounal of Plant Nutrition and Soil Science*)

Determination of Zinc in Rice Grains by DTZ Staining Technique and ImageJ Software Analysis

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Abstract

An efficient way to overcome the problem of malnutrition for a major part of the world's population is to increase Zn concentration in rice, which can be achieved through biofortification. Dithizone (DTZ) staining is a rapid, simple, and inexpensive method that allows the histochemical localization of labile Zn in different organisms. In this study, we showed how determination of Zn in brown rice could be made by dually implementing a staining technique and by using imageJ software to analyze the staining intensity. For that, we have used six upland rice (*Oryza sativa* L.) accessions widely cultivated in Brazil: Esmeralda, Line MG 1097 6, Relâmpago, Caravera, Line CMG 1510, and Curinga. The intensity zinc staining with DTZ (*I)* generated from ImageJ software provided a variable distribution in the concentration of Zn within and between rice grains. Different localization of Zn in different grain regions was indicated by the staining intensity index (*Y*) calculated from *R+G+B/3* values, weighted by the area of each level of staining (YAW). The concentration of Zn in brown rice varied in multiple regression analysis, showing major differences in YAW for the embryo, endosperm, and aleurone, especially in the endosperm and aleurone regions due to a large portion of the area in the kernel (r^2= 0.75, $p < 0.05$). The total YAW of those 3 regions presented the variation of total Zn concentration in the brown kernel among six rice accessions and was positively correlated with Zn concentration in brown kernel by chemical analysis (r^2 = 0.74, $p < 0.05$). We suggested that using this simple staining technique together with imageJ software analysis reveals a significant improvement in estimating the concentration of Zn in rice by taking into account the area of different grain regions with different Zn concentration. Moreover, it might be used for quick screening of large number of germplasms.

Key words: *Oryza sativa* / Zinc deficiency / Staining Intensity / Biofortification.

1 Introduction

Zinc (Zn) is one of the most important micronutrients required for normal and healthy growth in plants, animals, and humans (*Broadley* et al., 2007; *Alloway*, 2008). Zinc deficiency occurs in about half of the world's population, which can cause generalized problems associated with growth and development including delayed growth, stunted growth in children, increased susceptibility to infectious diseases, as well as birth defects in pregnant women (*Cakmak*, 2008; *Prasad*, 2009; *Graham* et al., 2012; *Prasad,* 2012). Indeed, consumption of agricultural products with inadequate Zn levels is considered to be the leading cause Zn deficiency. Therefore, increasing zinc concentration in crops such as rice, an important source of energy for more than three billion people living in Asia, could serve as a promising strategy for decreasing the incidence of zinc deficiency (*White* and *Broadley*, 2005; *White* and *Broadley*, 2009; *Bouis* and *Welch*, 2010).

Significant variation in grain Zn concentration can be found among different rice genotypes. The concentration of Zn ranged between 15.9 and 58.4 mg kg^{-1} for 939 brown rice samples evaluated at International Rice Research Institute (*Graham* et al., 1999) and from 13.32 to 43.65 mg Zn kg^{-1} in 274 samples from China's germplasm (*Jiang* et al., 2008). This impressive genotypic variation has led to a suggestion that by manipulating through plant breeding, one could more effectively produce rice abundant in Zn (*Welch* and *Graham* 2004; *Bouis* and *Welch*, 2010). Also, by distinguishing different parts of the rice grain (embryo, endosperm and aleurone layer) and determining each section's Zn concentration, scientists can more easily determine what causes variation in Zn concentration amongst various rice genotypes.

Recent studies have reported variations of Zn concentration in different parts of the grain. The highest Zn concentration (179 mg kg^{-1}) was found in the embryo, followed by the aleurone (51 mg kg^{-1}) and the endosperm 21 mg kg^{-1} (*Saenchai* et al., 2012). The variation in Zn concentration among different grain tissues affects the concentration in the whole kernel in rice (*Hansen* et al., 2009; *Saenchai* et al., 2012) similarly to wheat (*Cakmak* et al., 2010). Both grains show a high concentration of Zn especially in the endosperm which accounts for the largest portion of the grain. Therefore, a technique that takes into account the variation among the tissues and their contribution to whole grain Zn concentration should be useful in germplasm evaluation and breeding. This would be more convenient compared to chemical analysis when dealing with a large germplasm and would also allow the focus to be made on specific tissues, especially the endosperm. Staining with dithizone (DTZ) demonstrated that Zn is mainly localized in the embryo and aleurone of wheat grain and it has been suggested as a rapid, simple, and inexpensive method to evaluate Zn concentration in different parts of a seed (*Ozturk* et al., 2006; *Velu* et al., 2008; *Shobhana* et al., 2013). DTZ is a Zn-chelating agent that identifies the reactive (chelatable) Zn fraction through an intense red color produced by Zn-dithizone complex (*Ozturk* et al., 2006; *Cakmak* et al., 2010). The method of Zn localization within the grain tissues has also been used in rice grain by scoring the staining intensity by observing it under the microscope (*Prom-u-thai* et al., 2010; *Jaksomsak* et al., 2014). However, due to the small size of these images, the aforementioned method does not take into account the variation in size of the different grain tissues, which influences the calculation of total Zn content of the whole grain. Other disadvantages of visual scoring and staining techniques include inconsistency, lack of precision and higher probability of human error. Evaluating the intensity of Zn in the grain tissues by using systematic tools will help to minimize errors. Moreover, the use of an intensity index weighted by

grain area will take all parts of the grain tissue into account and help determine for the overall Zn content of the whole grain.

This study evaluated the variation of Zn concentration in rice grain among accessions from Brazil by using both chemical and staining techniques. The intensity of DTZ staining was also evaluated using the ImageJ software as the analysis tool. This would be useful information in breeding program for high-Zn rice genotypes in the future.

Materials and Methods

2.1 Plant material and sample preparation

Seeds of six upland rice (*Oryza sativa* L.) accessions were provided by a cooperative improvement program of upland rice, developed in partnership by the partners EPAMIG (Agriculture and Livestock Research Institute), UFLA (Federal University of Lavras), and EMBRAPA (Brazilian Agricultural Research Corporation) (Tab. 1). A field experiment was initiated in 2011/2012 in a upland conditions at Value for Cultivation and Use (VCU) in the EPAMIG Experimental Station (EELA), in Lavras, MG, Brazil (latitude: 21º14' S; longitude: 45º00' W and elevation: 918.841 above msl) during the rice growing season. VCUs are made for a particular regional scope, or for one or more Brazilian states and have usually been applied in plant research for annual plants to select the best accessions based on genotypic value for cultivar recommendation (*Soares* et al., 2007). Rice plants were grown in sandy clay soil with additional irrigation at EELA. Soil physical and chemical analyses are represented in Table 2. The experimental design was a randomized complete block with three replications. Each plot consisted of five rows of 5.0 m, spaced 0.4 m apart and a density of 80 seeds per meter. Basal fertilization consisted of the mixture of 400 kg ha^{-1} of NPK fertilizer (08-28-16) + 0.5% Zn and top

dressing with 100 kg ha^{-1} of N (ammonium sulfate) was applied in two equal portions (25 and 45 days from sowing). Rice grains were harvested during full maturity stage for Zn analysis.

((Table 1))

((Table 2))

2.2 Chemical analysis

Whole grains from ten plants per plot were harvested and oven-dried at 65°C until the rice reached a constant weight prior to digestion and analysis. Grain samples were dehusked in a testing husker (model MT, SUZUKI) to obtain brown rice (unpolished). For Zn analysis, samples of grains (paddy and brown rice) were acid-digested with 4 mL of concentrated HNO_3 + 2 mL of concentrated $HClO_4$ (Sigma-Aldrich, Saint Louis, MO, USA) at 120°C for 1h and then at 220°C until $HClO_4$ fumes were observed. Total Zn concentrations in the samples were determined by atomic absorption spectrophotometry (AAS, PerkinElmer Inc., San Jose, CA, USA) (*Malavolta* et al., 1997) with three replications. Tomato leaves SRM 1573A and rice flour 1568A standards (National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the rice samples to ensure accurate and reliable analytical data.

2.3 Diphenylthiocarbazone (DTZ) staining

About twenty paddy rice seeds were dehusked manually, excised longitudinally along the seed surface by a scalpel, and then submerged in freshly-prepared DTZ solution, by dissolving 1,5-diphenyl thiocarbazone (Merck) (500 mg L^{-1}) in methanol (AR grade) for 30 min, as described previously (*Ozturk* et al., 2006). Samples were rinsed thoroughly in DDI water and blotted dry using tissue paper.

2.4 Image acquisition and processing

Longitudinal sections of Zn-stained rice seeds were visualised at a 6X magnification with a Stereoscopic Zoom Microscope (Nikon SMZ 1500, Japan) and then images were obtained using a Camera Control Unit (Nikon, SU-1). 24 bit-depth images were analyzed on a desktop or laptop computer using ImageJ software (*Ferreira* and *Rasband*, 2012) Plugins Graphics RGB Profile Plot. The intensity of staining was measured through the RGB color space (Red, Green and Blue) defined by formula staining intensity values = (*R+G+B/3*). Intensity data represented the relative density of Zn in the grains, and was scored from 1 (less intense color), 2 (medium intense color), 3 (intense color) to 4 (very intense color) in accordance with the intensity of staining (RGB values, scale from 0 to 255).

2.5 Staining intensity analysis

Staining intensity values (*R+G+B/3*) from RGB images were evaluated by ImageJ software and then scored as 1, 2, 3 or 4, according to frequency distribution in 4-class interval as following in Eq. 1:

$$\text{score 1} = [\text{Min}(R+G+B/3) - (R+G+B/3)_1 >;$$

$$\text{score 2} = [(R+G+B/3)_1 - (R+G+B/3)_2 >;$$

$$\text{score 3} = [(R+G+B/3)_2 - (R+G+B/3)_3 >;$$

$$\text{score 4} = [(R+G+B/3)_3 - \text{Max}(R+G+B/3) >; \quad (1)$$

Where Min*(R+G+B/3)* and Max*(R+G+B/3)* are minimal and maximal staining intensity values (*R+G+B/3*), respectively, measured by ImageJ software analysis.

The Equation 2 was adapted according to the equation used by *Pintasen* et al. (2007) to calculate the staining intensity index among different seed lots of upland rice germplasm. Therefore, to calculate the staining intensity index (*Y*) within each region (embryo, endosperm or aleurone) of the rice seeds, Eq. 2 was used:

$$Y = \overbrace{\frac{(\%SEm \times SEm)}{100}}^{\text{Embryo}} \text{ or } \overbrace{\frac{(\%SEnd \times SEnd)}{100}}^{\text{Endosperm}} \text{ or } \overbrace{\frac{(\%SAl \times SAl)}{100}}^{\text{Aleurone}} \quad (2)$$

Where the percentage of the score region (%SEm = Embryo; %SEnd = Endosperm and %SAl = Aleurone): % Score region is represented by: 25% (score 1), 50% (score 2), 75% (score 3) and 100% (score 4). Unit of score region (SEm; SEnd and SAl) = 1: less intense color; 2: medium intense color; 3: intense color; 4: very intense color.

The index weighted by stained area of each region (*YAW*) was obtained by multiplying the staining intensity index (*Y*) within each stained region determined by the imageJ software with their area in mm^2, i.e., *YAW* = (*Y* region x area) and Total YAW = Σ[(*Y* embryo x area) + (*Y* aleurone x area) + (*Y* endosperm x area)].

2.6 Statistical analysis

Analysis of variance was carried out among the treatments using SISVAR version 5.3 software and coefficient of the variation was also calculated to estimate the reproducibility of the results (*Ferreira*, 2010). Differences between treatments were compared by LSD test ($p < 0.05$). Certain sets of data were also subjected to linear correlation and regression analysis, and also tested for their significance by measuring the correlation coefficient and determination.

Results

3.1 Concentration and localization of zinc in rice grains

A certain variation of Zn concentration was found in six Brazilian upland rice accessions for both brown rice (husks removed), which ranged from 27.2 to 36.5 mg kg^{-1}, and paddy rice (husks intact), from 29.9 to 43.0 mg kg^{-1} (Fig. 1). Considering Zn concentration in brown rice, which is the edible portion after dehusking process, the highest Zn concentration was found in accession 5 (36.5 mg kg^{-1}), whereas accession 4 had the lowest (27.2 mg kg^{-1}).

The DTZ staining showed comparable intensity of Zn localization when viewing the whole intact kernels (Fig. 1B), but the staining intensity of each grain region was more clearly differentiated in the longitudinal sections of the kernels (Fig. 1C), which was chosen for staining intensity analysis of rice grain among different regions and accessions through ImageJ software.

((Figure 1))

3.2 Staining intensity through image analysis

The images of each stained section were rescored using the staining intensity values (*R+G+B/3*). The ImageJ software was used to analyze the *R+G+B/3* values from RGB images in different regions of rice seed studied (embryo, endosperm and aleurone zones) (Fig. 2). There were different levels of staining intensity (intensity x distance) among regions of the seed (Fig. 2A; Fig. 2B and Fig. 2D), yet they were in concordance with the sensitivity of red tones perceptible to the human eye. The significant difference of the *R+G+B/3* value was found among grain regions of each rice accession (Fig. 3). The *R+G+B/3* values from RGB images observed in the seed regions ranged between 82.87

and 131.64 among the rice accessions studied. In most rice accessions, the highest *R+G+B/3* values were found in the embryo region, except for accession number 5, which presented the highest *R+G+B/3* value in the endosperm. The *R+G+B/3* values were used to calculate the staining intensity index (*Y*) as in Eq. 2.

((Figure 2))

((Figure 3))

The staining intensity index (*Y*) showed a wide variation of Zn concentration among seed regions (the embryo, endosperm, and aleurone) of 6 accessions (Fig. 4). In accession 1, 3, and 4, the highest index was found in the embryo compared to the aleurone and endosperm regions, while the highest index for the endosperm and aleurone regions was found in accession 5. There was no difference of the index among the 3 regions in accession 6. The area of each stained region was different within and between accessions (Fig. 5). The stained area of the endosperm was about 10 times larger than of the embryo and aleurone regions in all accessions. It was highest in accession 1, 4, 5, and 6 and lowest in accession 2 and 3. The YAW was used to represent the staining intensity of each region in the whole kernel. The concentration of Zn in brown rice varied with YAW of each grain region in a multiple regression (Eq. 3).

Brown rice [Zn] = 29.134 + 0.531 YAWaleurone - 0.192 YAWembryo + 0.104 YAWendosperm

(r^2= 0.755, $p < 0.05$) Eq. 3

Combining the YAW for the 3 regions gave whole grain YAW that indicated significant variation in the amount of Zn in the grain among the 6 accessions

studied ($p < 0.05$) (Fig. 6). Accession 5 had the highest whole grain YAW and the lowest was found in accessions 1, 2, 4, and 6. The total YAW among 6 accession was positively correlated with Zn concentration in brown rice kernel by chemical analysis ($r^2 = 0.74$, $p < 0.05$) (Fig. 7).

((Figure 4))

((Figure 5))

((Figure 6))

((Figure 7))

Discussion

4.1 Concentration and localization of zinc in rice grains

The chemical analysis showed a variation of Zn concentration in the kernel of 6 rice accessions studied, which was reported previously in several germplasms (*Graham* et al., 1999; *Saenchai* et al., 2012). The variations of Zn in grains among rice accessions may probably depend on the ability of Zn to mobilize from one part of the plant to the others such as from the husk to the grain. Recently, *Yoneyama* et al. (2010) reported that Zn redistribution for the grains and husks might be via the phloem after mobilization from the flag leaf and leaves below the flag leaf. However, several mechanisms may affect Zn remobilization such as the plant's nitrogen nutrition, the micronutrient transporters genes (e.g., ZIP family), the pH of the phloem sap and importantly, the forms of Zn-chelators as nicotianamine (NA) and deoxymugineic acid (DMA) in the plant (*Cakmak* et al., 2010; *Bashir* et al., 2012; *Clemens* et al., 2013). Mediated by *NAS* genes, the NA plays an important role in the intercellular and long-distance transport of Zn and has also contributed to Zn mobilization into the endosperm (*Takahashi* et al., 2009; *Clemens* et al., 2013). However, it has also been indicated that Zn concentration in the grain was

affected by the proportion of Zn in different grain regions both in rice (*Hansen* et al., 2009; *Saenchai* et al., 2012) and wheat (*Cakmak* et al., 2010). Thus, localization intensity of Zn in different grain regions is a key factor for Zn concentration in the whole kernel of rice. The DTZ staining of longitudinal grain section indicated that Zn is mostly localized in the embryo and aleurone layer parts, which agrees with previous investigations in several kinds of cereal grains by using the similar staining method (*Ozturk* et al., 2006; *Velu* et al., 2008; *Cakmak* et al., 2010; *Shobhana* et al., 2013). Therefore, to increase Zn concentration in brown rice kernel, attention should be carefully paid on Zn concentration in other plant/grain parts. In the grain tissues of rice, the staining intensity observed by human eyes can be difficult to distinguish, especially when dealing the narrow variation among grain tissues.

4.2 Assessing staining intensity through imageJ analysis

The easy and rapid method to assess grain Zn concentration would provide essential insight into decision-making for a breeding program for high Zn density in rice grains. Recently, the analysis of Zn partitioning and the localization of minerals in grain regions (embryo, endosperm, and aleurone layer) using different procedures have been suggested as a mediate instrument to screen grain Zn among large germplasms (*Takahashi* et al., 2009; *Saenchai* et al., 2012; *Lu* et al., 2013; *Kyriacou* et al., 2014). Staining with DTZ is one of the most promising screening methods that have been applied to assesses grain Zn in wheat (*Ozturk* et al., 2006), rice (*Prom-u-thai* et al., 2010; *Jaksomsak* et al., 2014), pearl millet (*Velu* et al., 2008), and maize (*Shobhana* et al., 2013). However, the staining intensity is usually counted through the scoring from the naked eye of observers and can easily result in error, depending on many factors such as gender, age, time and temperature. Our investigation was the first approach to assess Zn concentration in grains and the staining intensity in rice

grains by using DTZ staining method together with ImageJ software analysis. The chemical analysis was also carried out along with staining method to confirm the findings from our investigation. The ImageJ analysis program was selected as the tool to measure the staining intensity in rice grain by DTZ because it is simple and can be used by many people. Moreover, the program is freely accessible. The measurement of staining intensity (*I*) by ImageJ software analysis as demonstrated by profile of intensity was calculated to the staining intensity index (*Y*) for the comparable of the intensity level as investigated in the previous studies (1: less intense; 2: medium intense; 3: intense; 4: very intense) (*Pintansen* et al., 2007; *Jaksomsak* et al., 2014). When we used these indices, our results of rice staining showed that the embryo region of the grain's surface had the highest Zn concentration compared with the endosperm and aleurone layer in most accessions. This is consistent with recent findings of advanced method, which revealed a similar distribution pattern of Zn in the embryo of rice grains by Synchrotron X-ray fluorescence microscopy (XFM) (*Takahashi* et al., 2009; *Lu* et al., 2013; *Kyriacou* et al., 2014). However, the XFM technique may not be as easily available or accessible compared to the staining method addressed in this essay together with the ImageJ software. Evidence of intensive redistribution of Zn during seed germination in the embryo region proves that this region acts as a major source of nutrition in rice grains (*Ozturk* et al., 2006; *Saenchai* et al., 2012). Previous studies reported that the distribution of Zn was considerably localized in the embryo and aleurone layer of wheat (*Ozturk* et al., 2006) and rice kernels (*Lu* et al., 2013). However, the higher YAW index in the endosperm compared with the other regions of accessions 5 and 6 were probably due to the much larger endosperm surface area in those grains, demonstrated by the images from our analysis.

4.3 Correlation analysis

The significant variation of Zn concentration in brown rice kernel was determined by using in a multiple regression analysis and was complemented by measuring of the staining intensity weighting by stained area of the embryo, endosperm, and aleurone regions. This showed that various regions of the grain contained differentiated amounts of Zn concentration. The contribution was greater from the endosperm and aleurone regions due to their larger area compared with embryo in the image analysis. However, the concentration of Zn in all regions contributes to the total Zn concentration in the whole kernel as it was observed from the relationship between total Zn concentration in the kernel and the total YAW (r^2= 0.7417, $p < 0.05$). Therefore, our methodology indicates that manipulating the ImageJ software together with the DTZ staining can be efficiently used to assess the variation of Zn concentration between rice accessions and within the grain tissues from seeds, e.g., the endosperm and aleurone layer. It is a rapid, easy and economical method to evaluate Zn concentration in rice grain, especially when dealing with large germplasms (*Velu* et al., *2008*; *Shobhana* et al., 2013). This method allows plant breeders to determine the relative concentrations of Zn in rice within and between seed lot when working with rice samples that are genetically diverse (*Prom-u-thai* et al., 2003; *Pintasen* et al., 2007). However, thorough and specific evaluations of the small regions such as the embryo or the scutellum would be necessary to improve this method for further research and development.

Conclusions

This study confirms that the variation of Zn concentration can be distinguished among rice accessions, seed lots, and grain tissues by using DTZ staining together with the ImageJ software analysis. The staining method is easy, rapid, and economically viable and ImageJ software is freely accessible imaging

program. This is an important finding for rice breeders interested in developing high-density Zn genotypes to solve the problem of Zn deficiency among the world's population. The screening procedure can be carried out even in large number of samples without the problem of small amount per sample of rice, a problem that often occurs when performing chemical analysis. However, this method can be further refined by calibrating the staining surface to identify more closely the contribution of different tissues to total grain Zn.

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Table 1: List of the upland rice accessions used to determine grain Zn concentration by DTZ staining using imageJ software to analyze the staining intensity and chemical analysis.

Identification	Accessions (release year)[1]	Recommendations[2] (Brazilian States)
1	BRS Esmeralda (2012)	MT, GO, MA, MG, PA, PI, RO, RR, TO
2	Line MG 1097 6	-
3	BRSMG Relâmpago (2007)	MG
4	BRSMG Caravera (2007)	MG
5	Line CMG 1510	-
6	BRSMG Curinga (2004)	MG, GO, MT, MS, TO, AM, RO, MA, PA, PI

1 Accessions included 4 cultivars and 2 lines of upland rice.

[2] Refers to some locations of the regions where the cultivars or lines are tested and then suggested for growing.

Table 2: Soil physical and chemical analyses from EPAMIG Experimental Station, Lavras, Minas Gerais, Brazil.

Soil		Soil	
pH H_2O (1:2.5 ratio)	6,5	base saturation (%)	60,34
P[a] (mg dm^{-3})	14,09	Zn[a] (mg dm^{-3})	6,07
K[b] (mg dm^{-3})	96,00	Fe[a] (mg dm^{-3})	60,25
S[c] (mg dm^{-3})	10,78	Mn[a] (mg dm^{-3})	22,75
Ca[b] ($cmol_c$ dm^{-3})	2,80	Cu[a] (mg dm^{-3})	0,72
Mg[b] ($cmol_c$ dm^{-3})	0,90	B[e] (mg dm^{-3})	0,33
Al[b] ($cmol_c$ dm^{-3})	0,00	organic matter[f] (g kg^{-1})	19,9
H+Al[d] ($cmol_c$ dm^{-3})	2,59	Argila[g] (g kg^{-1})	460
effective CEC ($cmol_c$ dm^{-3})	3,95	Silte[g] (g kg^{-1})	60
CEC pH 7.0 ($cmol_c$ dm^{-3})	6,54	Areia[g] (g kg^{-1})	480

[a] Melich-1 method (0.05 mol L^{-1} + 0.0125 mol L^{-1}).. [b]KCl 1 mol L^{-1}. [c] Acetic - =acid monocalcium phosphate. [d] SMP method. [e] Hot water extraction. [f] $Na_2Cr_2O_7$ 0,67 mol L^{-1} + H_2SO_4 5 mol L^{-1} (*Embrapa*, 1999). [g] Granulometry was analyzed by the dispersion method (*Ruiz*, 2005).

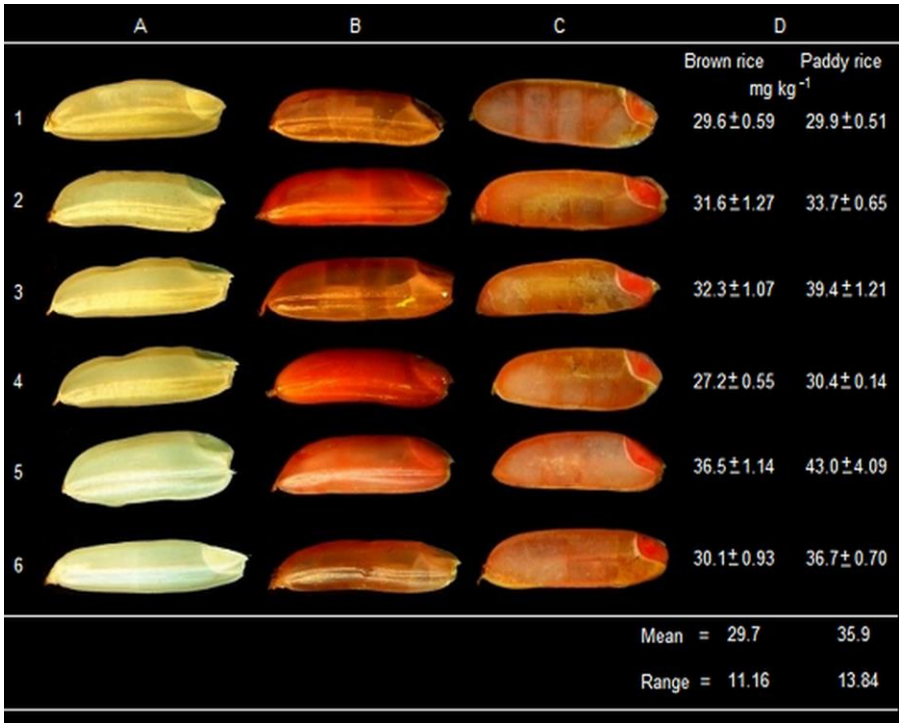

Figure 1: Stereomicrograph of whole grain non-stained (A), stained (B) and longitudinally-cut grain surface stained (C) by dithizone (DTZ) and Zn concentration (mg kg^{-1}) in brown and paddy rice (D). The relationship of Zn concentration between the brown and paddy rice in all rice accessions: $y = 0.53x + 12.57$ ($r = 0.86$, $p < 0.001$). Values shown are means ± SD of three independent replications. The intensity of staining represented the relative density of Zn in the grains.

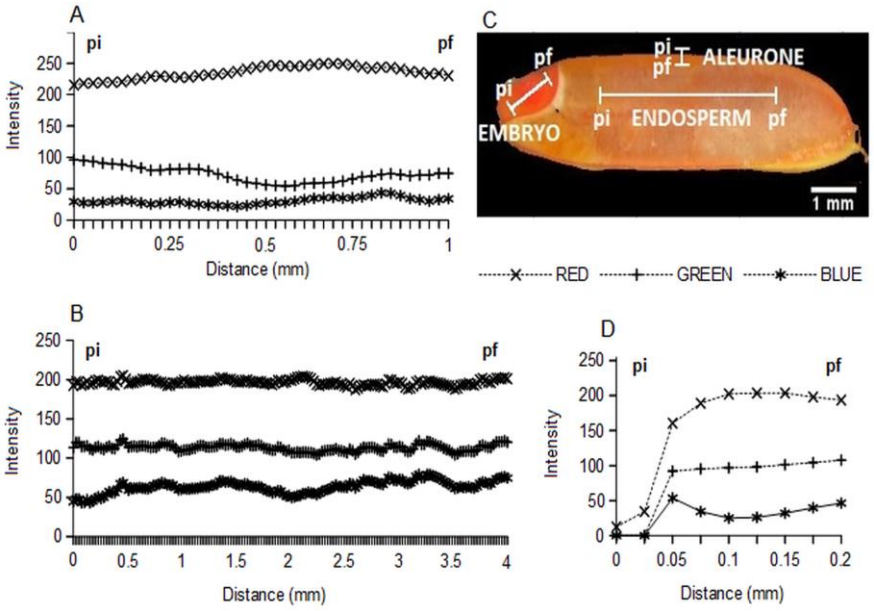

Figure 2: Stereomicrograph of longitudinally-cut grain surface stained by dithizone (DTZ). The intensity of staining represented the relative density of Zn in the grains. All graphics represented the intensity curves according to different seed regions studied such as embryo (A), endosperm (B) and aleurone (D) by ImageJ software. The longitudinal section of the grain kernel (C) shows the measurement area of the grain. Points: initial (pi) and final (pf).

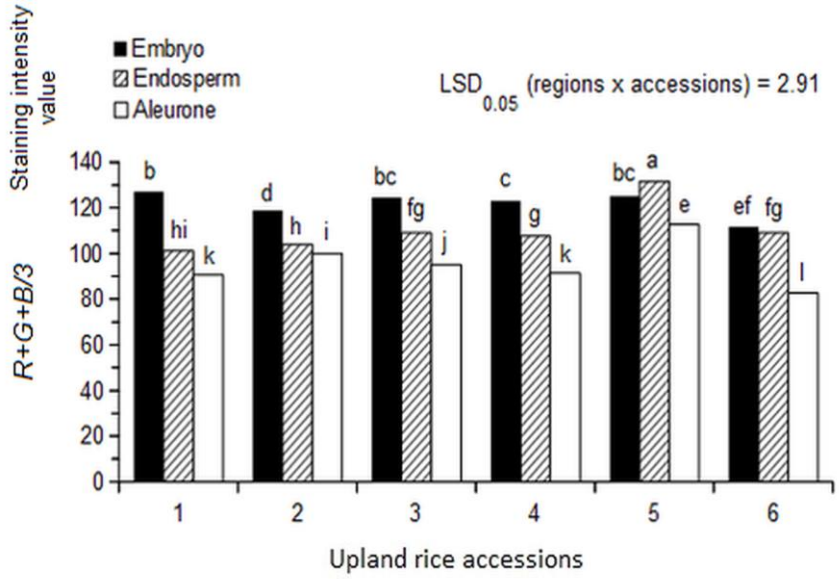

Figure 3: The staining intensity values among grain regions of 6 upland rice accessions from Brazil. Values shown represent means (bars) calculated from three independent replications. The staining intensity calculated from *R+G+B/3*. The RGB values were measured by imageJ software.

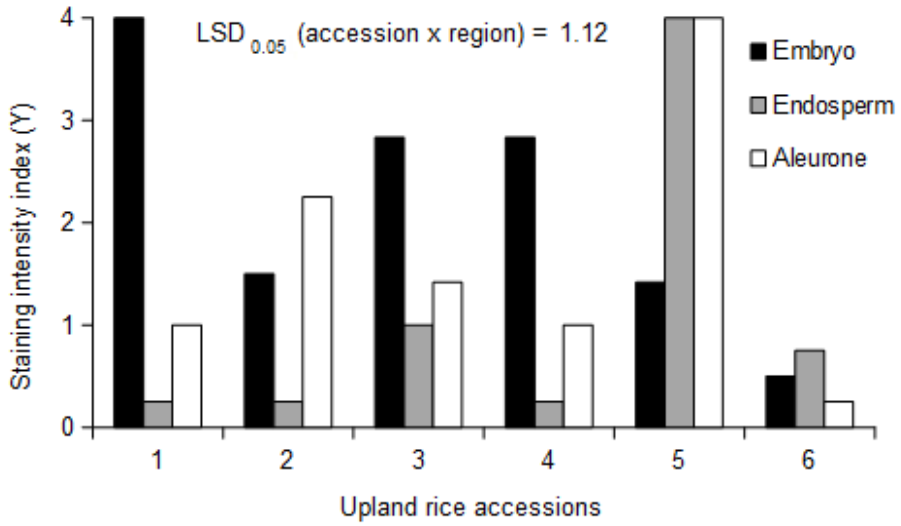

Figure 4: The staining intensity index (*Y*) among grain regions of 6 upland rice accessions from Brazil. Values shown represent means (bars) calculated from three independent replications. *Y* = [(percentage of each score region/100) x (score or staining of each region)].

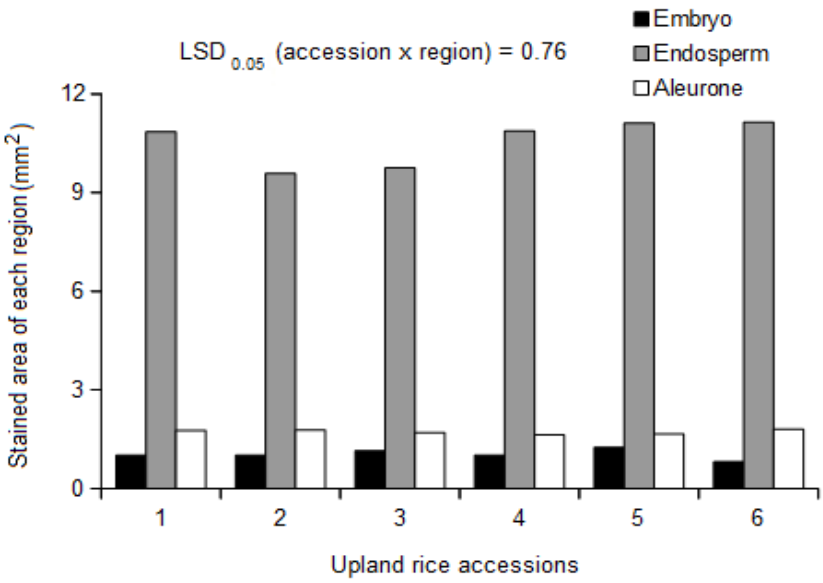

Figure 5: The stained area of each grain regions of 6 upland rice accessions from Brazil. Values shown represent means (bars) calculated from three independent replications.

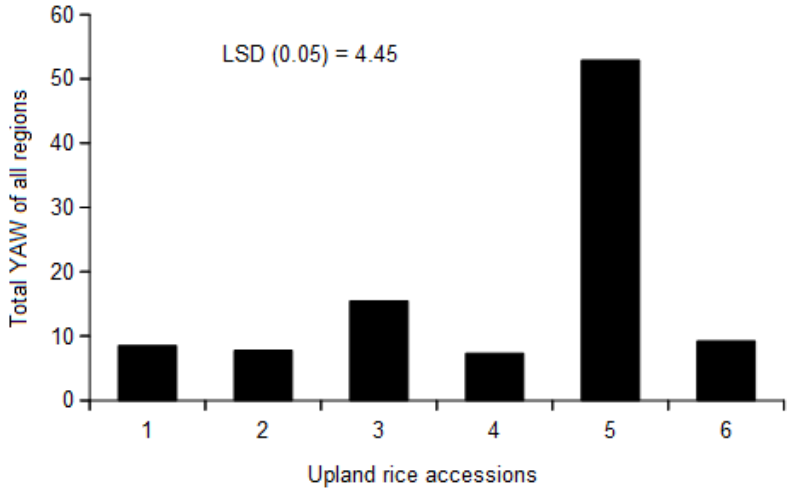

Figure 6: Total YAW of rice kernel from 6 upland rice accessions. Total YAW is the sum of the staining intensity index weighted by stained area of each region (*YAW*) in the embryo, aleurone layer and endosperm as in the following equation: Total YAW = Σ[(*Y* embryo x area) + (*Y* aleurone x area) + (*Y* endosperm x area)].

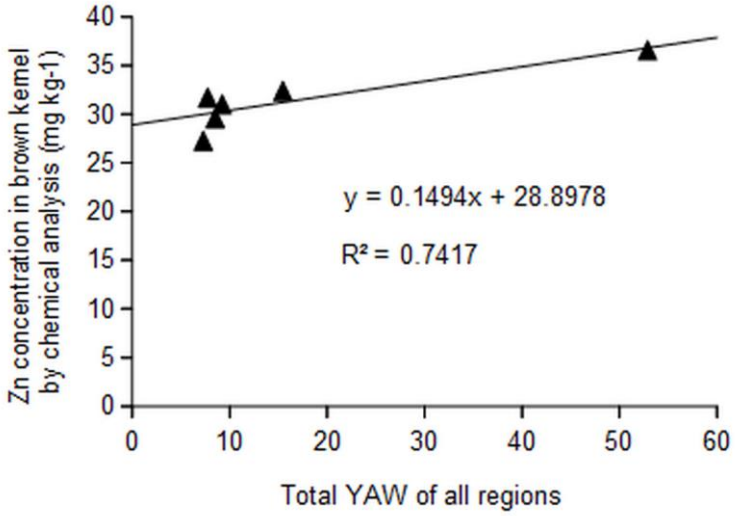

Figure 7: Relationship between Zn concentration in brown rice from chemical analysis and total YAW of all regions from 6 upland rice accessions. Total YAW is the sum of the staining intensity index weighted by stained area of each region (*YAW*) in the embryo, aleurone layer and endosperm as in the following equation: Total YAW = Σ[(*Y* embryo x area) + (*Y* aleurone x area) + (*Y* endosperm x area)].

ARTIGO 2

Characterization of Zn homeostasis related genes in rice genotypes (*Oryza sativa* L.) with different seed Zn densities

(Normas de publicação da revista *Rice*)

Characterization of Zn homeostasis related genes in rice genotypes (*Oryza sativa* L.) with different seed Zn densities

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Abstract

Background: Understanding mechanism of root uptake, translocation, and accumulation of Zn into the grains through physiological and molecular studies may be interesting to alleviate human malnutrition related to zinc deficiency. Thus, we have investigated how some genes involved in metal homeostasis can act through the gene expression during early tillering (ETS), full tillering (FTS), and anthesis stages (AS) and observed the relative contribution to Zn uptake and remobilization from plant tissues to grains by using of rice genotypes with different seed Zn concentration.

Results: Zn-sufficiency treatment promoted higher Zn concentration and plant development, especially when high Zn density genotypes were used. Moreover, Zn supply affected significantly the grain Zn concentration (paddy rice) and total Zn content in high density genotypes than low Zn density genotypes. Expression profiles from sqPCR analysis showed overexpression of *Os*ZIP1 and *Os*ZIP4 at ETS as well as *Os*ZIP3 and *Os*ZIP4 for both FTS and AS when different plant tissues (roots shoot and flag leaves) were analyzed. At ETS, *Os*ZIP1 showed

higher expression profiling especially in shoots, whereas *Os*ZIP4 was more expressed both in root and shoots under Zn deprivation. qPCR analysis revealed higher relative expression in rice plants grown under Zn deficiency (at ETS) especially in roots, whereas higher relative expression was found under Zn-sufficiency (at FTS and AS).

Conclusion: Our study revealed Zn-efficient genotypes especially low Zn density genotypes. Under Zn-sufficiency treatment significant higher Zn content observed in whole grains from high Zn density genotypes was influenced by root-to-shoot translocation of Zn. Expression analysis by sqPCR allowed identification of *Os*ZIP4 as the prevalent gene in all plant growth stages, i.e., it is most closely related to Zn homeostasis. qPCR analysis showed differences in expression levels in rice tissues from plants grown under Zn treatments. However, considering gene expression analysis, which mostly is made in plants in early development, these results are in accordance with most approaches found in literature. Further investigation using a large set of representative genotypes for studies with biofortification as well as identifying physiological traits to Zn accumulation in grains may be necessary.

Keywords: Rice; Zinc; Zn Biofortification; Translocation; Remobilization; Zn efficiency; Zn density genotypes; Gene expression; Transporters;

Background

Rice (*Oryza sativa* L.) is one of the world's most important staple food for human nutrition and it is being widely cultivated for consumption in south and southeast Asia as source of energy, but unfortunately it is perceived as a cereal grain poor in important micronutrients such as iron (Fe), zinc (Zn), and vitamin A when compared with other cereals (Bouis and Welch 2010; Graham et al. 2012; Bhullar and Gruissem 2013; Miller and Welch 2013). Therefore, the consumption of a cereal-based diet is closely related to major causes of micronutrient malnutrition in humans (i.e., Zn deficiency), especially in some developing countries where rice grain is a primary source of Zn intake and other rich sources of Zn is limited (Pfeiffer and McClfferty 2007; Sharma et al. 2013). Zn is an important cofactor for over 300 enzymes that are involved in significant functions such as RNA and DNA metabolism, protein synthesis, gene expression, carbohydrate metabolism, fertility and seed production, and disease resistance (Chapasis et al. 2012; Prasad 2012). Thus, an adequate intake (recommended daily reference, RDA) of Zn required for a normal growth and adult development, i.e., from 8 to 14 mg of Zn per day (Trumbo et al. 2001), is the way to decrease Zn-deficiency incidences, which is estimated to affect nearly 50% of the world's population (Hotz and Brown 2004; Welch and Graham 2004).

Among the strategies to alleviate Zn deficiency, plant breeding through genetic biofortification was suggested as the most easily applicable, better cost-effective and affordable in the target populations (White et al. 2012a, b). Several studies have emphasized the identification of physiological traits influencing Zn-deficiency tolerance as well as to investigate if it affects the translocation and remobilization of minerals from source tissues to rice grains (Jiang et al. 2007; Jiang et al. 2008; Wu et al. 2010; Sperotto et al. 2012; Impa et al. 2013). However, there is a lack of information about the contribution of Zn uptake and remobilization from plant tissues to grains in different growth stages. Indications of the effect of Zn supply on its remobilization among rice genotypes in different growth stages are still divergent and unclear. For instance, rice plants grown under sufficiency or surplus conditions of Zn during post-flowering stage were reported with root uptake as the main Zn accumulation source in the grains (Jiang et al. 2007). Similar results were also found in rice plants grown under Zn-sufficiency condition at the grain-filling stage, whereas, under Zn-deficiency condition, simultaneous uptake and remobilization of Zn to grains were observed among different genotypes (Impa et al. 2013). On the other hand, Wu et al. (2010) reported that the remobilization of Zn from source tissues to grains occurs at maturity and not directly from root uptake during grain-filling stage, which explained why Zn mobility within rice plants is more essential than the root uptake capacity (Wu et al. 2011). Based on the previous explanation, Zn

accumulation in rice grains was found in different pathways, which would be clearer to understand if the experiment on genes expression analysis associated with the Zn homeostasis would be included.

The expression patterns of numerous genes in plants have been widely studied to gain a better understanding of molecular mechanisms involved in metals homeostasis such as Fe and Zn mediated by transporters induced by different Zn supplies (Ishimaru et al. 2005; Suzuki et al. 2008; Ishimaru et al. 2011; Milner et al. 2013). In rice crops, metal transporters (e.g., ZIP family members) have been one the most important in processes such as the soil Zn uptake, the root-to-shoot Zn translocation and Zn transportation to seeds, although other mechanisms (i.e., metal-chelating complexes) can also act in the regulation of the metal uptake and transport throughout plants (Ramesh et al. 2003; Grotz and Guerinot 2006; Masuda et al. 2008; Suzuki et al. 2008; Bashir et al. 2012). Moreover, studies where metal transporters genes are highly expressed suggest that these genes may be associated with the Zn efficiency in rice plants (Chen et al. 2008; Milner et al. 2013). Therefore, approaches involving gene expression together with partitioning of plant tissues in different growth stages are still very poorly known. It could be an important tool for biofortification. Hence, in this study, the aims were: (i) to indicate how genes involved in metal homeostasis can act through the gene expression during different rice plant stages; and, (ii) to determine the relative contribution of Zn

uptake and remobilization from plant tissues to grains in rice genotypes with different seed Zn concentration.

Methods

Plant materials and growth conditions

Four upland rice (*Oryza sativa* L.) genotypes, two of them high seed Zn concentration genotypes (Canastra and KPK) and two low seed Zn concentration genotypes (Sertaneja and RD21) were derived from Brazil and Thailand from previous field screening (Table 1). KPK (high Zn and low yield) and RD21 (low Zn and high yield) genotypes are Thai genotypes (Jaksomsak et al. 2014), whereas Canastra (high Zn) and Sertaneja (low Zn) are Brazilian genotypes used in this study. Seeds were surface-sterilized in 0.5% NaOCl for 20 min, rinsed and germinated on petri dishes containing germination paper soaked with distilled water for 8 days (4 days in a dark room at 25-28ºC and 4 days in room temperature). After germination, 8-day-old seedlings were suspended in the nutrient solution by floating Styrofoam boats into plastic containers. The full-strength nutrient solution had the following composition (adapted from Sperotto et al. 2012): 1 mM $Ca(NO_3)_2.4H_2O$, 3 mM KNO_3, 25 µM $CaCl_2.2H_2O$, 0.5 mM KH_2PO_4, 0.75 mM K_2SO_4, 0.5 mM $MgSO_4.7H_2O$, 10 µM H_2BO_3, 9.5 µM $MnCl_2.4H_2O$, 0.5 µM $CuSO_4.5H_2O$, 0.5 µM $(NH_4)_6Mo_7O_{24}.4H_2O$, 0.1 µM $NiCl.6H_2O$, and 50 µM FeNaEDTA. The adaptation periods were carried out by

settings seedlings 5 days each in the 25%, 50% and 75%-strength solution. Then, the adapted seedlings were transferred into two different solution pots Zn concentrations, 0.005 µM (Zn-deficiency) and 1.5 µM $ZnSO_4.7H_2O$ (Zn-sufficiency) as suggested by Impa et al. (2013). The flowering and harvesting days were recorded as shown in Table 1. The day the plants were transferred to full-strength nutrient solution was considered as day 0 (0 days after planting - DAP). The nutrient solution was replaced every 5 days and the pH was adjusted to 5.5±0.5 using either HCl or NaOH. Plants were grown in a growth chamber under controlled environment with 16 h at 28°C light and 8 h at 23°C dark. Relative humidity was maintained at 75% and photon flux density of 270 µmol m^{-2} s^{-1}.

Table 1 Characteristics of rice genotypes used in the experiment.

Rice genotypes	Origin	Zn density genotype	Grain Zn concentration (µg g DW^{-1})		Days to 50% flowering (DAP)[1,2]	Days to harvest (DAP)
			brown rice	paddy rice		
Canastra	Brazil	high	34.9	40.6	+Zn = 98; -Zn = 177	+Zn = 173; -Zn = 229
Sertaneja	Brazil	low	17.6	16.8	+Zn = did not flower; -Zn = 91	+Zn = did not yield; -Zn = 184
KPK	Thailand	high	27.0	36.7	+Zn = 98; -Zn = 105	+Zn = 184; -Zn = 184
RD21	Thailand	low	10.6	11.6	+Zn = 143; -Zn = 143	+Zn = 225; -Zn = 225

[1] DAP = Days after planting. [2] Zn treatments conditions as suggested by Impa et al. (2013): -Zn = Zn-deficiency (0.005 µM); +Zn = Zn-sufficiency (1.5 µM).

Sampling and measurements

For RNA extraction and Zn analysis, different plant parts (root, shoot and flag leaves) were separated during early tillering, full tillering, and anthesis stages (Counce et al. 2000). The same stages of growth plant were used for all genotypes. At full maturity stage, seeds were also harvested for Zn analysis. A chlorophyll meter (Minolta CO., LTD. Japan) was used to obtain SPAD values on the last fully expanded leaf from rice plants grown at 20 DAP and 40 DAP. Length of shoot (at 20, 28, 40 and 76 DAP) and roots (at 28 and 76 DAP) were measured from the bottom node of the stem to a tip of the longest shoot and root, respectively. For each stage, three samples were collected from one plant with multiple tillers and samples were pooled. Three biological replicates were collected to be used with the semi-quantitative and quantitative PCR studies.

Zn quantification by AAS

The collected plant parts (root, shoot and flag leaves) were thoroughly washed with deionized water. Seed samples were separated manually by hand to yield brown rice and paddy rice. All samples were oven dried at 60°C for 72 h. For Zn analyzes, samples were homogenized and weighed (±0.5 g) before acid-digestion with 10 mL of the mixture nitric acid + perchloric acid (6:1). Digestions were performed using a heating block with an exhaust-collecting manifold. Concentrations of Zn were determined by atomic absorption

spectrometer (AAS, model Z-8230, Hitachi, Tokyo, Japan). Tomato leaf standards (SRM 1573A, National Institute of Standards and Technology, Gaithersburg, MD) were digested and analyzed along with the rice samples to ensure accuracy of the instrument calibration.

RNA extraction and cDNA synthesis

Total RNA was isolated from rice tissues (root, shoot and flag leaves) using an easy-RED^{TM} Total RNA Extraction Kit (Intron Biotechnology, Inc., Seoul, Korea) and treated with 1 U of DNase I, RNA-free (Thermo Scientific, #EN0521). cDNA was prepared using Tetro cDNA Synthesis Kit (Bioline Reagents Ltd., London, UK), following to the manufacturer's instructions. First-strand cDNA synthesis was synthesized from 2 µg of total RNA using reverse transcriptase (Bioline Reagents Ltd., London, UK).

Semi-quantitative RT-PCR analysis

Semi-quantitative RT-PCR analysis (sqPCR) was carried out to confirm the differential expression of the 4 genes involved in metal homeostasis (*Os*ZIP1, *Os*ZIP3, *Os*ZIP4 and *Os*NAC5) in rice plants. The primers for sqPCR analysis (Additional file 1: Table 1S) were designed to amplify between 80 to 150 bp and to have similar Tm values (60±2) using Primer3 (v. 0.4.0) software (http://frodo.wi.mit.edu/, Rozen and Skaletsky, 2000) and OligoAnalyzer 3.1

program (http://www.idtdna.com/, Integrated DNA Technologies, Inc, Coralville, IA). The rice UBQ-5 (AK061988) rice gene was used as internal control (Jain et al. 2006).

The sqPCR was carried out with the OnePCR™ *Plus* pre-mixed solution (GeneDirex Inc., Taipei, Taiwan) containing Taq DNA polymerase, PCR buffer, dNTP, gel loading dyes, enhancer, and fluorescence dye according to the manufacturer's instructions. In a final volume of 25 µL were used 2 µL of cDNA aliquot (5-fold dilution) and 0.2 µL of each primer (20 µM). The reaction conditions for the PCR were an initial denaturation time of 2 min at 94°C, followed by 35 cycles of PCR (20 s at 94°C, 20 s at 60°C, 20 s at72°C), and a final time of 10 min at 72°C. Amplified products were visualized on a 2% TBE agarose gel. Bands were photographed using the LED transilluminator (BLOOK, GeneDirex Inc., Taipei, Taiwan). To obtain the reproducibility of semi-quantitative RT-PCR, the experiment was repeated 2 times with two independent biological replicated samples.

Quantitative RT-PCR and data analysis

Quantitative RT-PCR analysis (qRT-PCR) were carried out in an iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) using the same primers as described by semi-quantitative RT-PCR analysis. The amplification mixture to a final volume of 20 µL was composed of 10 µL of 2x

SensiFAST SYBR No-ROX Mix (Bioline Reagents Ltd., London, UK), 0.4 µL of each primer (20 µM), 7.2 µL of DEPC water and 2 µL of cDNA template (diluted 1:100). PCR cycling conditions were composed of an initial polymerase activation of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 15 s at 72°C. Samples were held for 2 min at 40°C and then heated from 55 to 99°C with a ramp of 0.5°C/0.5 s to acquire data to produce the melting curve of the amplified products. Gene expression was evaluated using the comparative C_T (threshold cycle) by $2^{-\Delta C_T}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). All samples were analyzed in three technical replications, a ΔC_T value was obtained by subtracting the UBQ-5 C_T value from the C_T obtained of the target gene. Each data point corresponds to three true biological replicate samples.

Statistical Analysis

All data were submitted to analysis of variance (ANOVA) using SISVAR version 5.3 software (Ferreira 2010). Differences between treatments were compared in two-way by the least significant difference ($P \leq 0.05$). Pearson's correlation analyses were carried out using two significance levels ($P \leq 0.05$ and 0.01).

Results

Zinc concentration, SPAD values and plant growth in rice genotypes

To check the contribution of different Zn supplies on the parameters of Zn concentration and plant growth we used two rice genotypes grown in nutrient solution (Impa et al. 2013). At early tillering stage, rice plants grown under Zn-sufficiency conditions presented higher Zn concentration in both shoots and roots with a little variation between genotypes (Fig. 1A and B). Shoot and root length increased in KPK genotype when plants were grown in the presence of 1.5 µM Zn (Fig. 1E and F), which may also have been favorable to better plant development (Fig. 1C and D). Zn concentration under Zn-sufficiency condition was tightly represented in both shoots from KPK (131.7 µg g DW^{-1} of Zn, high Zn genotype, Fig. 1A) and roots from RD21 (131.7 µg g DW^{-1} of Zn, low Zn genotype, Fig. 1B). Additionally, regarding to shoots (0.88 g $plant^{-1}$) and roots (0.38 g $plant^{-1}$) dry weight as well as the total Zn content per plant our results also showed at the same tendency as described for Zn concentration for the same rice genotypes (Additional file 3: Table 3S). Shoot Zn concentration ranged from 28.8 to 29.5 µg g DW^{-1} and 109.8 to 131.7 µg g DW^{-1} under Zn-deficiency and Zn-sufficiency conditions, respectively. Moreover, root Zn concentration ranged around 34 µg g DW^{-1} under Zn-deficiency and from 95.9 to 122.5 µg g DW^{-1} under Zn-sufficiency condition.

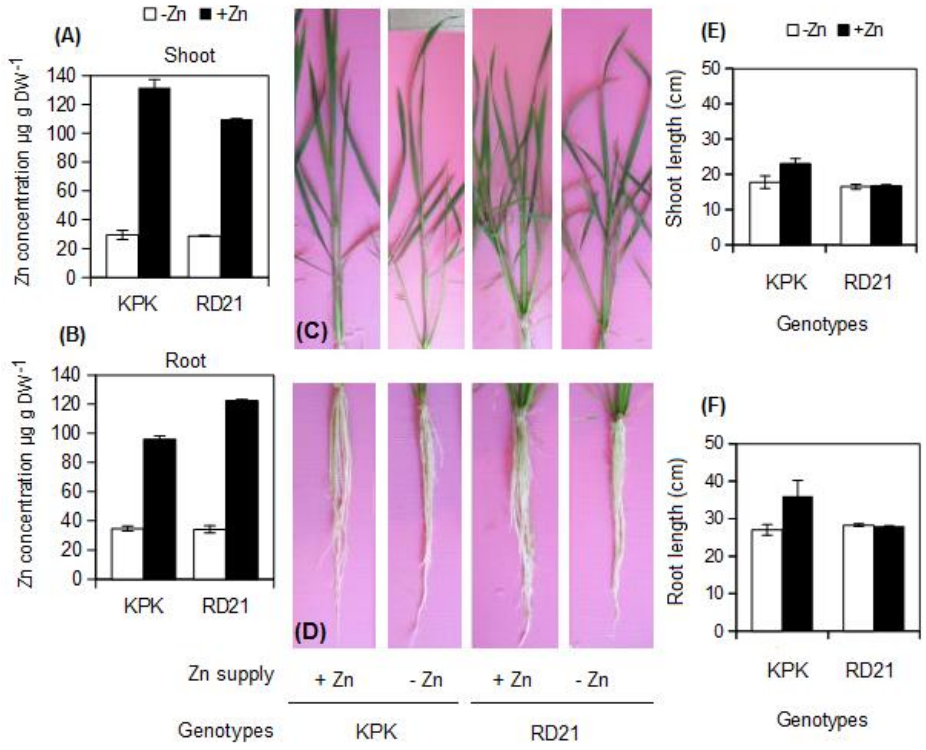

Figure 1 Zinc concentration and rice plant development under different Zn conditions. Concentration of Zn in shoots (A) and roots (B), rice growth (C and D) and length of shoots (E) and roots (F) from Thai rice genotypes at early tillering stage. Samples from shoots and roots of rice under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions. Genotypes classified according to seed Zn-density (Table 1). Data were recorded with 3 and 5 triplicates for Zn concentration and plant length, respectively. Error bars represent standard deviation.

The SPAD values were slightly higher in the KPK rice genotype. However, differences of the visual symptoms of Zn deficiency on flag leaves were observed only between the Zn treatments, but not between genotypes as shown in Figure 2A and B. In addition, there were little or no statistical differences in the SPAD values and shoot length at the late growth stages (Additional file 2: Table 2S).

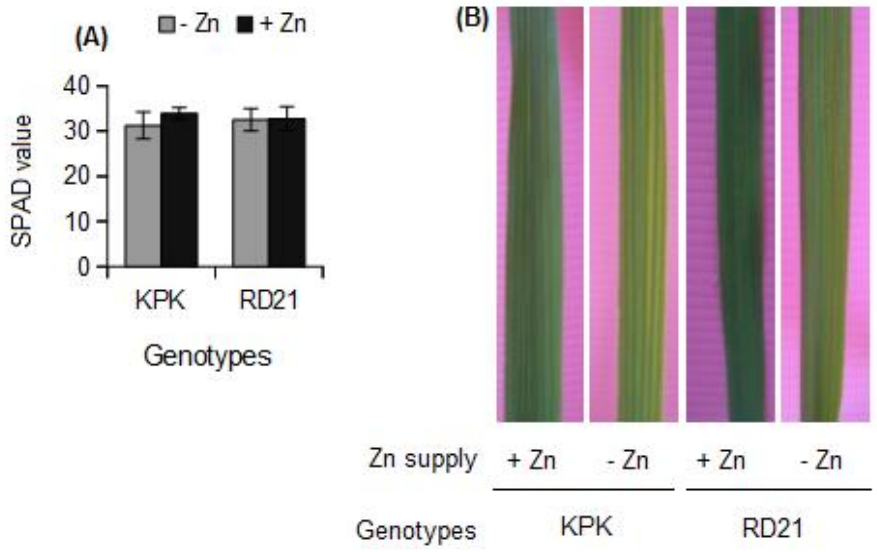

Figure 2 Morphological characteristics of plants grown under different Zn conditions. SPAD value (A) and flag leaves (B) from rice genotypes under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions genotypes at anthesis stage. Error bars represent standard deviation.

Zn concentration and total Zn content in the rice grains

At full maturity, rice grains were harvested for Zn analysis. There were differences of Zn concentration in both brown and paddy rice, as well as on the total Zn content in the whole grains of different rice genotypes under different Zn supplies (Table 2). From analysis of variance, all measurements (Zn concentration and total Zn content) were significantly affected by genotypes (G), Zn treatments (Zn), and by the interaction G x Zn, except for Zn concentration in brown rice, where no statistical difference on Zn treatments was found. Under Zn-sufficiency conditions, higher Zn concentration and total Zn content were found in the high Zn density genotypes, Canastra and KPK, respectively. In addition, under Zn-deficiency conditions higher Zn concentration (brown and paddy rice) and total Zn content were noticed in all low Zn density genotypes (Sertaneja and RD21) when compared with high Zn density genotypes, although some results did not present statistical differences. Moreover, highest representative increases in Zn concentration were found on Canastra grains (high Zn density genotype) grown under Zn supply, e.g., Zn concentration was 3.1 fold higher in the brown rice and 3.5 fold higher in paddy rice under Zn-sufficient than that under Zn-deficiency conditions. Furthermore, significantly higher total Zn content (1.75 µg $grain^{-1}$) was observed in the whole grains from KPK (high Zn density genotype) than the others rice genotypes tested.

Table 2 Concentration and total content of Zn in rice grains under Zn-deficiency (-Zn) and Zn-sufficiency (+Zn) conditions.

Genotypes	Zn concentration (µg g DW^{-1})				Total Zn content (µg $grain^{-1}$)	
	brown		paddy		whole grain	
	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn
Canastra	18.13d	56.31a	17.22e	60.17a	0.43d	1.27b
Sertaneja[a]	45.16abc	-	38.89cd	-	0.93c	-
KPK	29.25cd	43.16abc	30.54d	49.92b	0.88c	1.75a
RD21	38.97bc	51.90ab	38.03cd	47.10bc	0.95c	1.07bc
Mean	32.88	50.46	31.17	52.40	0.80	1.36
Genotype (G)	7.22*		23.31***		35.45***	
Zn treatment (Zn)	1.99[ns]		13.38**		14.74**	
G x Zn	25.22***		65.48***		51.06***	
CV (%)	19.93		12.18			

Values shown represent means for two different replications. ANOVA was performed in two-way on LSD test. Different letters within the same kind of rice

(brown, paddy or whole grains) are significantly different between different Zn treatments at $p<0.05$. ***, **, * and ^{ns} indicate significant difference at $p<0.001$, 0.01, 0.05 and non-significant, respectively. [a] Did not flower under Zn-sufficiency condition.

Semi-quantitative RT-PCR (sqPCR)

To examine the differential expression of four genes related to zinc homeostasis (*Os*ZIP1, *Os*ZIP3, *Os*ZIP4 and *Os*NAC5) at three different stages of plant growth such as early tillering (ETS), full tillering (FTS), and anthesis (AS), tissues (root, shoot, and flag-leaves) from rice genotypes were analyzed by semi-quantitative RT-PCR (sqPCR) analysis (Figure 3, 4 and 5). At ETS all genes were expressed in all tissues (roots or shoots) examined as well as under different Zn treatments (Fig. 3A and B). However, the *Os*ZIP1 demonstrated higher expression profiling (high intensity levels measured by grayscale values, Fig. 3B) especially in shoots (no significant differences between Zn treatments were found), whereas *Os*ZIP4 was more expressed in both shoots and roots under Zn-deficiency conditions. Differences of expression levels were not affected by the rice genotypes as well as by different Zn treatments.

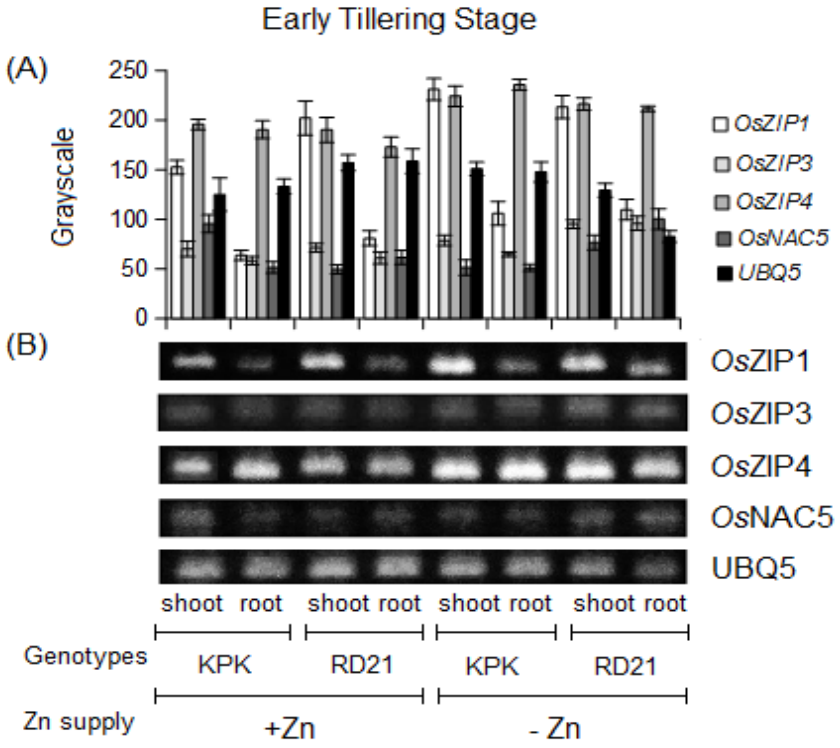

Figure 3 Expression patterns of genes involved in zinc homeostasis (*Os*ZIP1, *Os*ZIP3, *Os*ZIP4 and *Os*NAC5). Semi-quantitative RT-PCR analysis (sqPCR) in roots and shoots from rice plants under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at early tillering stage (A and B). Ubiquitin gene (UBQ5) of rice was used as an internal control. The band intensities through the grayscale levels analyzed by imageJ software (A) reported the differences in gene expression among the genes by sqPCR analysis. Error bars represent standard deviation.

The sqPCR analysis in shoots from rice plants at full tillering stage (FTS) showed differential expression levels among rice genes. Lower expression levels were found in the genes *Os*ZIP1 and *Os*NAC5, whereas higher expression values were observed in the genes *Os*ZIP3 and *Os*ZIP4 according to the intensity values (Fig. 4A) and profile of the gel bands (Fig. 4B). Under Zn-deficiency condition detectable expressions were clearly better observed than those in rice plants grown under Zn-sufficiency condition. Moreover, there were no noticeable comparative expression levels between studied rice genotypes.

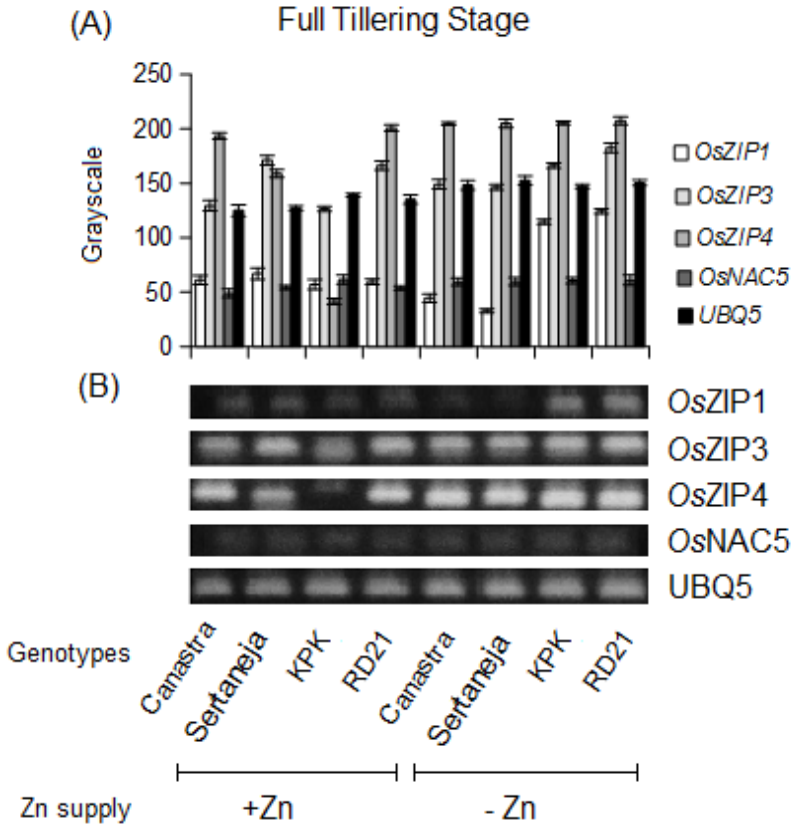

Figure 4 Expression patterns of genes involved in zinc homeostasis (*Os*ZIP1, *Os*ZIP3, *Os*ZIP4 and *Os*NAC5). Semi-quantitative RT-PCR analysis (sqPCR) of genes in shoots from rice plants under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at full tillering stage (A and B). Ubiquitin gene (UBQ5) of rice was used as an internal control. The band intensities through the grayscale levels analyzed by imageJ software (A) reported the differences in

gene expression among the genes by sqPCR analysis. Error bars represent standard deviation.

Gene expression profiling through sqPCR from flag-leaf tissues at anthesis stage (AS) showed very low (*Os*NAC5) or high expression among genes evaluated (*Os*ZIP1, *Os*ZIP3 and *Os*ZIP4) (Fig. 5). The analysis revealed that *Os*ZIP3 was the gene most expressed (intensity values above 200, Fig. 5A), i.e., it is a specific gene of late growth stage. The *Os*ZIP4 was the gene with second higher expression except for RD21 genotype under Zn-sufficiency conditions where *Os*ZIP1 presented higher expression level.

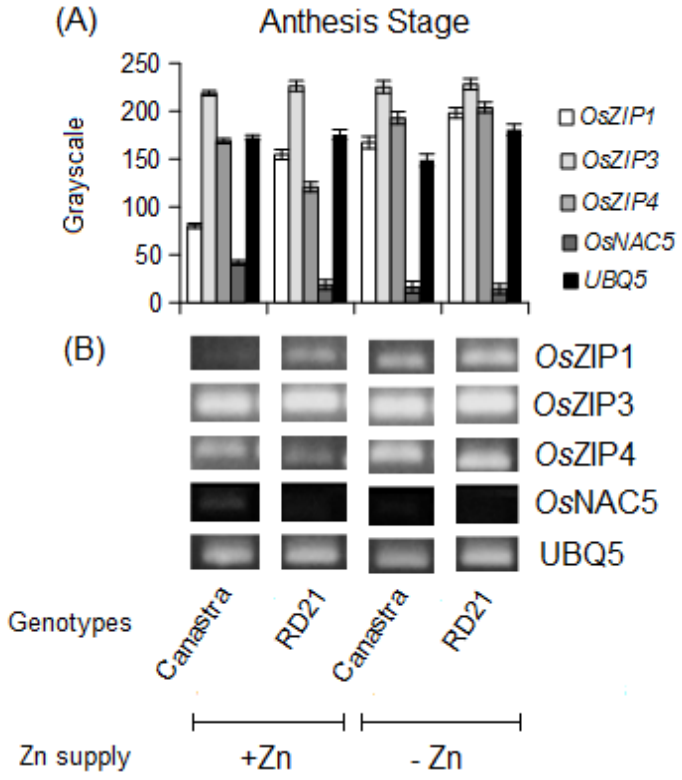

Figure 5 Expression patterns of genes involved in zinc homeostasis (*Os*ZIP1, *Os*ZIP3, *Os*ZIP4 and *Os*NAC5). Semi-quantitative RT-PCR analysis (sqPCR) of genes in flag leaves from rice plants under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at anthesis stage (A and B). Ubiquitin gene (UBQ5) of rice was used as an internal control. The band intensities through the grayscale levels analyzed by imageJ software (A) reported the differences in

gene expression among the genes by sqPCR analysis. Error bars represent standard deviation.

Quantitative RT-PCR (qPCR) analysis

We used the two highest expressed genes from sqPCR at different plant growth stages (ETS, FTS and AS) to investigate its expression pattern by quantitative RT-PCR (qPCR) analysis (Fig. 6, 7 and 8). At ETS, higher relative expressions (*Os*ZIP1 and *Os*ZIP4) were found in rice plants grown under Zn-deficiency (0.005 µM Zn) when compared to Zn-sufficiency (1.5 µM Zn) condition for both roots and shoots (Fig. 6A, B, C and D). Although there was no statistical differences between Zn treatments or between rice genotypes (KPK and RD21), numerically the relative expression from roots prevailed over shoots. Regarding the rice genotypes, the relative expression was identical in both KPK and RD21 for all evaluated genes.

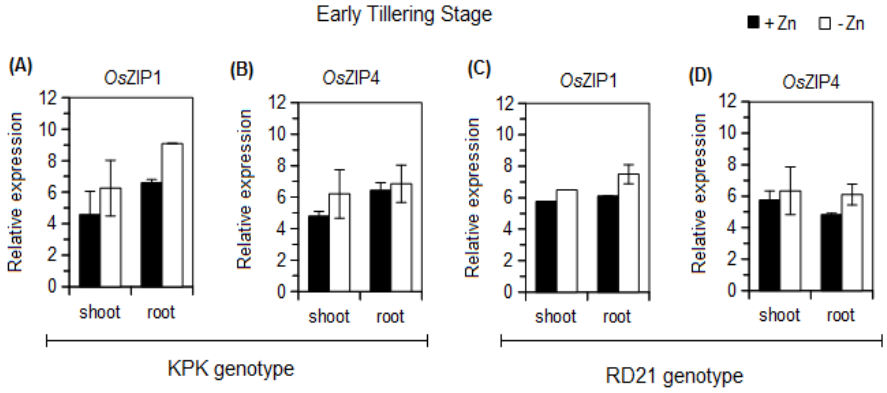

Figure 6 Relative expression of genes involved in zinc homeostasis (*Os*ZIP1 and *Os*ZIP4). Quantitative RT-PCR analysis of genes in roots and shoots from KPK (A and B) and RD21 (C and D) rice genotypes under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at early tillering stage. Ubiquitin gene of rice was used as an internal control. The data are the means of three replications. Error bars represent standard deviation.

At FTS, qPCR analysis using shoot tissue from four rice genotypes showed differential expression for both *Os*ZIP3 and OsZIP4. Considering Zn treatments, higher expression levels were found under Zn-sufficiency in all rice genotypes except for the KPK and RD21 genotypes when *Os*ZIP3 gene was analyzed. Moreover, the *Os*ZIP3 had better relative expression in both Canastra and Sertaneja genotypes under Zn-sufficiency conditions and KPK and RD21 genotypes under Zn-deficiency conditions (Fig. 7A), however the standard

deviation make it a little unclear. On the other hand, low variation on the relative expression was found in *Os*ZIP4 where it was weakly higher under Zn-sufficiency than Zn-deficiency conditions in three of the four rice genotypes tested (Fig. 7B).

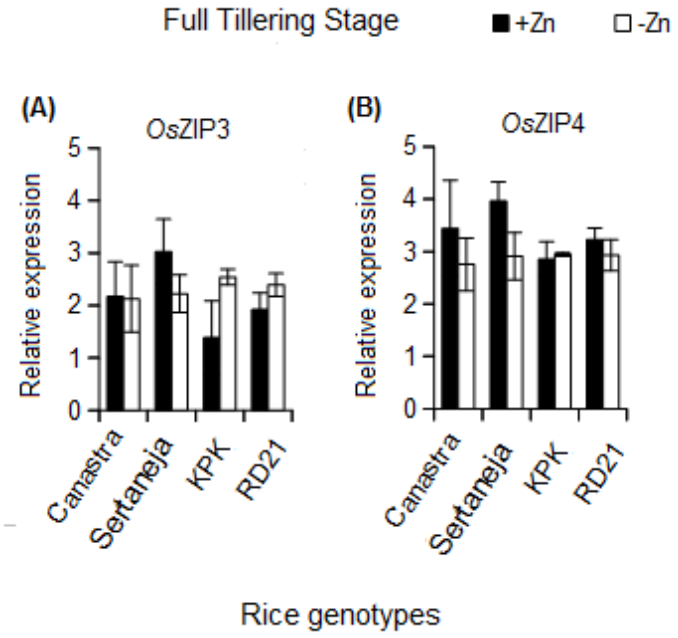

Figure 7 Relative expression of genes involved in zinc homeostasis. Quantitative RT-PCR analysis of *Os*ZIP3 (A) and *Os*ZIP4 (B) in shoots tissues from four rice genotypes under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at full tillering stage. Ubiquitin gene of rice was used as an internal control. The data are the means of three replications. Error bars represent standard deviation.

To observe whether the expression from qPCR is also expressed in late growth stages (AS) we used two rice genotypes (Canastra and RD21) (Fig. 8). Higher expression was presented for *Os*ZIP4 (Fig. 8B) when compared to *Os*ZIP3 (Fig. 8A) in flag leaves tissues, however, both genes in most of the rice genotypes were overexpressed under Zn-sufficiency conditions.

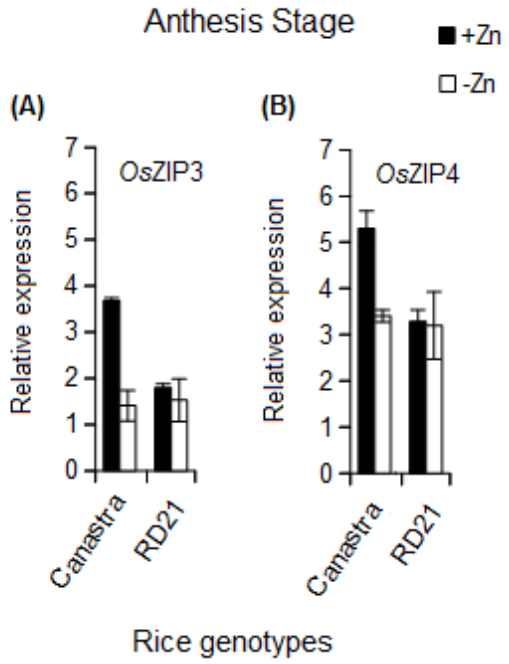

Figure 8 Relative expression of genes involved in zinc homeostasis. Quantitative RT-PCR analysis of *Os*ZIP3 (A) and *Os*ZIP4 (B) in flag leaves tissues from two rice genotypes under Zn-sufficiency (+Zn) and Zn-deficiency (-Zn) conditions at anthesis stage. Ubiquitin gene of rice was used as an internal

control. The data are the means of three replications. Error bars represent standard deviation.

Discussion

In many regions of the world, approximately half of cereal crop areas are soils with low Zn levels, which results in significant losses in crop growth and yield as well as in decreasing the grain Zn concentration (Suzuki et al. 2006; Alloway 2008; Cakmak 2008). As expected, under the conditions used in this study, rice plants grown under Zn-deficiency conditions could present lower development and shown some Zn-deficiency symptoms on leaves as well as lower Zn concentration in their tissues than that under Zn-sufficiency conditions. When we investigated rice genotypes with different Zn density in their grains, our results showed that high Zn density genotype in comparison with the low Zn density genotype promoted higher Zn concentration and plant length at early tillering stage. It is important for improving human nutrition through the choices of genotypes with high grain Zn density. Moreover, higher concentration and content of Zn in the rice grains from high Zn density genotypes (Canastra and KPK) (Table 2) show that these genotypes may respond to Zn supplementation due to higher root-to-shoot Zn translocation index (Additional file 3: Table 3S). On the other hand, higher Zn efficiency was found in low Zn genotypes such as Sertaneja (101.1%) and RD21 (59.7%) at ETS and Sertaneja (76%) at FTS

(Additional file 3: Table 3S), i.e., there seem to be potential for further biofortification approaches especially in Zn deprived. However, the use of more genotypes with different Zn densities in the grains from different regions or countries can be suggested in further studies. Similarly, Wu et al. (2010) also reported that the high Zn density genotype is more effective in Zn accumulation in several tissues at early growth stage of rice plants. These authors even suggest that high Zn density genotypes are more capable of transporting Zn from roots to shoots, i.e., it is closely associated with the ability of Zn translocation within plants.

Semi-quantitative RT-PCR (sqPCR)

In recent years several studies have demonstrated that translocation and remobilization of Zn from source tissues to grains depend on physiological traits and genotypic differences for Zn efficiency in rice plants (Grotz and Guerinot 2006; Wu et al. 2011; Sperotto et al. 2012; Impa et al. 2013). However, molecular mechanisms that explain the ability of the rice genotypes in to obtain certain quantities of Zn are still not totally understood. Results from literature have previously shown that the ZIP family plays an important roles as metal transporters (e.g., transport of Zn), which can be up-regulated in response to Zn deficiency or under high external Fe concentration (Grotz and Guerinot 2006; Ishimaru et al. 2006; Wang et al. 2013). Through the semi-quantitative RT-PCR,

our study revealed the expression of *Os*ZIP4 in all tissues as well as in plant growth stages (ETS, FTS and AS), whereas *Os*ZIP1 and *Os*ZIP3 were better expressed in tissues from rice plants at early and mainly late growth stages, respectively. That differential expression at different rice growth stages may be interesting to a better understanding of the genotypes more efficient which may be used for agronomic or genetic biofortification studies. The expression of *Os*ZIP4 also has been found by Ishimaru et al. (2005) in roots in response to Zn-deficiency at early growth stage. Moreover, the expression of *Os*ZIP3 and *Os*ZIP4 was found by Chen et al. (2008) who have suggested that genes may contribute to high Zn efficiency in rice. Regarding *Os*ZIP1, it seems to be up-regulated in root and shoots from rice grown under low Zn conditions (Ramesh et al. 2003; Ishimaru et al. 2005; Yang et al. 2009), which is in accordance with our results.

Quantitative RT-PCR (qPCR) analysis

To keep an adequate intracellular Zn availability, plant cells demand transport mechanisms that are engaged in Zn influx, efflux, and intracellular compartmentalization (Ishimaru et al. 2005). Thus, Zn-related genes are commonly identified as follows: member of the Zn-regulated transporter and a Fe-regulated transporter-like protein (ZIP), P-type metal ATPase (HMA), cation diffusion facilitator (MTP), and nicotianamine synthase (NAS) genes (Ishimaru

et al. 2008). Among the genes studied in our research, *Os*ZIPs family members (*Os*ZIP1, *Os*ZIP3 and *Os*ZIP4) were those that better represented Zn uptake and translocation due to higher expression levels in the root, shoot, and flag-leaves in different plant growth stages (Figs 6, 7 and 8). At ETS, all genes (*Os*ZIP1 and *Os*ZIP4) had higher expression in both root and shoot under Zn-deficiency conditions. Ishimaru et al. (2008) reported that *Os*ZIP1, *Os*ZIP3 and *Os*ZIP4 usually are transcriptionally up-regulated in both shoots and roots of Zn deficient rice. In rice crops, metal transporters as ZIP1 and ZIP3 appear as important in the uptake of Zn from soil under Zn deficiency whereas ZIP4 seems particularly essential for root to shoot translocation as well as for Zn transport to seed (Ramesh et al. 2003; Grotz and Guerinot 2006; Bashir et al. 2012). Regarding the middle and late stages (FTS and AS, Fig. 7 and 8), in general we observed higher expression level under Zn-sufficiency, except for the Thai genotypes (KPK and RD21, Fig 7A), where higher expression was found under Zn-deficiency conditions. These observations are in accordance with data found in the literature (Ramesh et al. 2003; Ishimaru et al. 2005; Ishimaru et al. 2007). Considering the standard deviation showed on expression level graphic (Figs 7 and 8) between the Zn treatments and the Zn-supply condition there was no change, i.e., the results are identical.

Conclusions

Our study revealed Zn-efficient genotypes (Sertaneja and RD21) that provided higher grain Zn concentration (brown and paddy rice) under Zn-deficiency conditions. On the other hand, under Zn-sufficiency conditions high Zn density genotypes (Canastra and KPK) had higher Zn content in their grains, which may also be suggested as important for further investigations involving biofortification. Expression analysis by sqPCR allowed identification of *Os*ZIP4 as the prevalent gene in all plant growth (early, middle and late) stages. Moreover, *Os*ZIP1 and *Os*ZIP3 were better expressed at early and late stage, respectively. At early tillering stage, relative expression analysis of genes identified by sqPCR (*Os*ZIP1, *Os*ZIP3 and *Os*ZIP4) and analyzed by qPCR showed higher expression levels in rice tissues (roots and shoots) of plants grown under Zn-deficiency. However, at middle and late plant growth stages, relative expressions levels had results opposite to the ones found at early tillering stage. Considering that gene expression analysis is mostly done in plants in early development, these results are in accordance most of the approaches found at the literature. Further investigation using a large set of representative genotypes for studies with biofortification as well as identifying physiological traits to accumulation of Zn in grains may be necessary.

Abbreviations

ETS: Early tillering stage; FMS: Full tillering stage; AS: Anthesis stage; DAP: Days after planting; AAS: Atomic absorption spectrometer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RFD and CP provided the seeds. RFD, CP and TP designed the experiments as well as coordinated the study. RFD and TP obtained the AAS and PCR analysis. RFD performed the statistical analyzes and drafted the manuscript. RFD, CP, TP and LVP prepared the final manuscript. All authors read and approved the final manuscript.

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Additional file 1

Table 1S Forward (F) and reverse (R) primers for Zn-related genes used for semi-quantitative RT-PCR and quantitative RT-PCR

Gene	Acession N°	Primer sequences (5'→ 3')	Product size (bp)	References
*Os*ZIP1	AY302058	F-GTGGAGGAACCTGTGGACGATC R-AAGGCGAGGGAGTAGACGAC	123	[1];[2]; [6] up-regulated in rice root and shoots under low Zn
*Os*ZIP3	AY275180	F-ACATCACCGTGGTCACCGTC R-TGGCTGTGTGAGTGCGAGTG	82	[1];[2];[3]; [6] up-regulated in rice root and shoots under low Zn
*Os*ZIP4	AB126089	F-GGCGACTTTCTTCTCCCTCACT R-TGAAGACTCCCTCGACGACAAG	110	[2]; [4] highly expressed under conditions of Zn deficiency in roots and shoots
*Os*NAC5	AK064292	F-GATCGGAGATTGAATGGAGTGCG R-TTGCGGCACAGGTAGTACATCA	102	[5] involved in metal homeostasis in flag leaves
UBQ5	AK061988	F-ACACCAAGCCCAAGAAGCAG R-GGCGTCGTCCACCTTGTAGAAC	80	[7] internal control gene in rice samples

[1] Ramesh et al. (2003); [2] Ishimaru et al. (2005); [3] Ishimaru et al. (2007); [4] Suzuki et al. (2012); [5] Sperotto et al. (2009); [6] Yang et al. (2009); [7] Jain et al. 2006.

Additional file 2

Table 2S SPAD index, root and shoot length from four upland rice genotypes under under Zn-deficient (-Zn) and Zn-sufficient (+Zn) conditions [a]

Days	Traits	Genotypes							
		Canastra		Sertaneja		KPK		RD21	
		-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn
20 DAP	SPAD	27.3d	36.6ab	36.8ab	37.7a	31.3c	33.9bc	32.6c	31.2c
	Shoot (cm)	10.7d	14.4abc	16.0ab	15.2abc	14.2bc	16.8a	13.3c	13.5c
28 DAP	Root (cm)	26.2cd	26.0cd	30.8b	23.7d	29.7bc	36.0a	27.8bcd	27.3bcd
	Shoot (cm)	15.8d	22.0a	19.3b	18.3bc	18.8b	22.7a	16.5cd	16.7cd
40 DAP	SPAD	32.0c	38.8ab	30.9c	41.7a	32.8c	37.9b	34.0c	38.4ab
	shoot (cm)	20.2c	23.7bc	25.8b	26.8ab	27.3ab	30.5a	20.7c	20.6c
76 DAP	Root (cm)	33.0b	23.2e	32.0bc	24.5e	30.2cd	39.8a	28.5d	25.2e
	Shoot (cm)	28.0cd	24.5de	31.5bc	33.7b	32.2b	40.7a	24.0de	23.83e

Values shown represent means for three different replications (n = 3). ANOVA was performed in two-way with at LSD test. Different letters within a row are significantly different between different Zn treatments at $p<0.05$. DAP = Days after planting.

Additional file 3

Table 3S Dry weight, zinc concentration, Zn content and efficiencies of Zn in the root and shoot from rice genotypes under Zn-deficient (-Zn) and Zn-sufficient (+Zn) conditions

Genotypes	Dry weight (g plant^{-1})				Zn concentration (mg kg^{-1})				Zn content (µg plant^{-1})				ZnE[1]	ZnTI[2]	
	Root		Shoot		Root		Shoot		Root		Shoot		(%)	(%)	
	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn	-Zn	+Zn		-Zn	+Zn
Harvest 1[a]															
Canastra	0.2	0.3	0.7	0.9	33.2	211.6	37.5	111.5	6.7	58.2	25.6	96.1	76.2	77.9	81.6
Sertaneja	0.2	0.2	0.6	0.6	33.6	254	29.9	122.8	8.0	59.7	18.3	75.3	101.1	69.8	56.5
KPK	0.1	0.3	0.4	0.9	34.7	95.9	29.5	131.7	7.1	32.3	17.2	115.6	45.8	71.7	78.0
RD21	0.2	0.4	0.5	0.8	34.1	122.5	28.8	109.8	8.1	46.4	15.3	89.3	59.7	65.6	65.7
G	ns		ns		ns		ns		ns		ns		-	***	
Zn	***		**		**		***		**		***		-	ns	
GxZn	*		ns		ns		ns		ns		ns		-	*	

Continue...

Harvest 2[b]															
Canastra	0.6	1.0	2.2	4.3	89.4	279.7	35.9	87.0	194.7	1098.3	21.1	72.1	50.5	59.9	57.3
Sertaneja	0.7	0.5	2.0	2.6	88.6	505.3	51.2	207.8	192.8	1308.7	30.0	96.5	76	62.2	69.7
KPK	1.0	1.7	6.1	12.0	88.6	400.4	43.2	139.9	174.3	4813.7	29.9	243.0	50.4	75.6	70.7
RD21	0.8	1.2	3.9	8.3	133.5	545.1	50.1	111.4	809.0	4509.8	47.9	130.4	47.5	64	59.1
G	nd		nd		****		***		nd		nd		nd		
Zn	nd		nd		****		****		nd		nd		nd		
GxZn	nd		nd		***		**		nd		nd		nd		

[a] Early tillering stage; [b] Fullt tillering stage. Values shown represent means for three different replications (n = 3). ANOVA was perfomed in two-way using at LSD test. ****, ***, **, * and [ns] indicate significant difference at $p \leq 0.0001$, 0.001, 0.01, 0.05 and non-significant, respectively. [1] Zn efficiency (ZnE) = (shoot dry weight at -Zn)/(dry weight at +Zn) x 100; [2] Root-to-shoot Zn translocation index (ZnTI) = (total shoot Zn content)/(total Zn content per plant) x 100.