Universidade Federal do Rio Grande do Sul - UFRGS
Instituto de Biociências e Departamento de Botânica
Laboratório de Fisiologia Vegetal

Caracterização de novos transportadores da família CDF envolvidos na homeostase de Zn e Mn em plantas de arroz (*Oryza sativa*)

Aluna: Paloma Koprovski Menguer

Tese submetida ao Programa de Pós-Graduação em Botânica da UFRGS como requisito parcial para a obtenção do título de Doutor.

Orientadora: Profa. Dra. Janette Palma Fett
Porto Alegre, Fevereiro de 2013

Agradecimentos

À minha orientadora Janette Palma Fett, pela oportunidade e confiança no meu trabalho, assim como, por oferecer um laboratório com ótima infra-estrutura e disponibilidade de todos os materiais necessários para o desenvolvimento dos projetos.

À minha co-orientadora Lorraine E. Williams, pela orientação e puxões de orelha; meu trabalho não teria tanta qualidade se eu não tivesse teu engajamento no projeto e confiança no meu trabalho.

Ao meu grande amigo Felipe Klein Ricachenevsky, responsável pela formação de uma pesquisadora empolgada, crítica e determinada. Tua influência me fez acreditar na carreira científica, a minha admiração pelo teu trabalho e pela tua pessoa é infinita. Muito obrigada por fazer parte da minha vida!

Aos colegas e amigos do laboratório de Fisiologia Vegetal da UFRGS, em especial aos queridos Raul Antônio Sperotto, agora profe Raul, e Karina Lopes.

Às flores do meu jardim, Carolina Ruedell, Joséli Schwambach, Lívia Scheunemann, Naíla Cannes e Variluska Fragoso, a nossa amizade é para sempre. Vamos envelhecer juntas tomando chá com cuca. Amo vocês.

Aos meus queridos amigos Lívia Kmetzsch e Charley Staats, pelas infindáveis conversas nos corredores da UFRGS, apoio científico e empréstimo de reagentes.

Aos meus pais, por toda base emocional e valores. Posso estar em qualquer lugar do mundo e enfrentar qualquer problema na vida se eu tiver o apoio de vocês. Meu amor é incondicional!

Ao meu irmão Rodrigo Koprovski Menguer, exemplo de garra, responsabilidade, trabalho e sucesso, tu é meu herói desde a infância e meu amor por ti não tem como ser medido. Obrigada por ter sempre apoiado essa irmã riponga!

Ao meu amor Gabriel Hofmann Selbach, passamos toda a difícil fase do doutorado juntos, ficamos muito tempo separados e nosso amor só cresceu. Obrigada por todo apoio e compreensão, tu me fez acreditar que um relacionamento pode ser leve e feliz, não preciso mais nada além disso, e como tu sempre diz, o importante é que vamos ficar juntos no final. Te amo!

Instituições onde o trabalho foi desenvolvido:

- Laboratório de Fisiologia Vegetal da Universidade Federal do Rio Grande do Sul, RS, Brasil.
- Williams Lab, University of Southampton, UK

Fontes Financiadoras:

- Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)
- Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)
- European Union Framework Programme 6, como parte do projeto: Integrated Project Public health impact of long-term, low level mixed element exposure in susceptible population strata (PHIME)

Resumo

A homeostase de metais é mantida nas células vegetais por transportadores especializados que compartimentalizam ou realizam o efluxo dos íons metálicos, mantendo as concentrações citosólicas dentro de uma faixa adequada. OsMTP1 e OsMTP11 são membros da família de transportadores de metais CDF/MTP em *Oryza sativa*, e acredita-se que eles possuem papéis importantes na homeostase de Zn e Mn em plantas de arroz, respectivamente. A complementação funcional do mutante de Arabidopsis *mtp1-1* pela expressão ectópica de *OsMTP1* demonstra que esta proteína transporta Zn *in planta*. Embora a localização subcelular de OsMTP1 tenha sido previamente caracterizada como de membrana plasmática, nossos resultados indicam localização no tonoplasto. A expressão heteróloga de *OsMTP1* em levedura mutante *zrc1 cot1* resgatou a hipersensibilidade do mutante a Zn; OsMTP1 também mitigou em algum nível a sensibilidade de *zrc1 cot1* a Co; e complementou a hipersensibilidade a Fe e Cd dos mutantes *ccc1* e *ycf1*, respectivamente, quando testados em baixas concentrações dos metais correspondentes. Esses resultados sugerem que OsMTP1 transporta Zn, mas também Co, Fe e Cd, talvez com afinidade mais baixa. Estudos com mutações sítio-dirigidas nos permitiram identificar duas substituições em OsMTP1, L82F e H90D, que parecem alterar a função de transporte de OsMTP1. OsMTP1ΔL82F ainda transporta baixos níveis de Zn, com afinidade aumentada pra Fe e Co, enquanto OsMTP1ΔH90D elimina completamente o transporte de Zn e aumenta o transporte de Fe. Além disso, mostramos evidências do papel de OsMTP11 no transporte de Mn. A expressão heteróloga de *OsMTP11* no mutante de levedura *pmr1* complementou parcialmente a hipersensibilidade deste a Mn. Identificamos quatro resíduos possivelmente envolvidos na função de transporte de OsMTP11: as substituições D267H, D162A e E213G reduziram o resgate da hipersensibilidade a Mn em diferentes níveis; enquanto L150S complementou totalmente a hipersensibilidade do mutante *pmr1*. Os resultados apresentados neste trabalho são novas contribuições para a área de nutrição mineral de plantas e podem ser úteis em aplicações biotecnológicas como fitorremediação e biofortificação.

Abstract

Heavy metal homeostasis is maintained in plant cells by specialized transporters that compartmentalize or efflux metal ions, maintaining cytosolic concentrations within a narrow range. OsMTP1 and OsMTP11 are members of the CDF/MTP family of metal cation transporters in *Oryza sativa*, and are believed to play important roles in Zn and Mn homeostasis in rice, respectively. Functional complementation of the Arabidopsis T-DNA insertion mutant *mtp1-1* demonstrates that OsMTP1 transports Zn *in planta*. Although OsMTP1 had been previously suggested to be targeted to the plasma membrane, results here indicate localization at the tonoplast. Heterologous expression of *OsMTP1* in the yeast mutant *zrc1 cot1* complemented the Zn-hypersensitivity of this mutant; OsMTP1 could also alleviate to some extent the Co sensitivity of *zrc1 cot1*; and rescue Fe and Cd hypersensitivity in *ccc1* and *ycf1* mutants, respectively, when tested at low concentrations of corresponding metals. These results suggest that OsMTP1 transports Zn but also Co, Fe and Cd, perhaps with lower affinity. Site-directed mutagenesis studies allowed us to identify two substitutions in OsMTP1, L82F and H90D, which appear to alter the transport function of OsMTP1. OsMTP1ΔL82F can still transport low levels of Zn, with enhanced affinity for Fe and Co, while OsMTP1ΔH90D completely abolishes Zn transport but improves Fe transport. In addition, we show evidence for a role of OsMTP11 in Mn transport. Heterologous expression of *OsMTP11* in the yeast mutant *pmr1* partially complemented the Mn-hypersensitivity of this mutant. It was identified four residues possibly involved in OsMTP11 transport function; D267H, D162A and E213G reduced the Mn-hypersensitivity rescuing capacity in different levels; and L150S fully rescued Mn-hypersensitivity in the *pmr1* mutant. The results presented here are novel contributions to the field of plant mineral nutrition and may be useful in future biotechnological applications such as phytoremediation and biofortification.

Lista de elementos químicos

Ca – *calcium* (cálcio)

Cd – *cadmium* (cádmio)

Co – *cobalt* (cobalto)

Cu – *copper* (cobre)

Fe – *iron* (ferro)

Mg – *magnesium* (magnésio)

Mn – *manganese* (manganês)

Ni – *nickel* (níquel)

P – *phosphorous* (fósforo)

Zn – *zinc* (zinco)

Lista de abreviaturas

ADN – ácido desoxirribonucleico

ATP – *adenosine triphosphate* (trifosfato de adenosina)

CAX – *calcium exchangers* (permutadores de cálcio)

CDF – *cation diffusion facilitator* (facilitador da difusão de cátions)

cDNA – *complementary DNA* (DNA complementar)

EGFP – *enhanced green fluorescent protein* (proteína fluorescente verde aprimorada)

FRO – *ferric reductase oxidase* (redutase oxidase férrica)

GFP – *green fluorescent protein* (proteína fluorescente verde)

His – *histidine* (histidina)

HMA – *heavy metal associated* (associado à metais pesados)

IRT – *iron-regulated transporter* (transportador regulado por ferro)

MT – *metalothionein* (metalotioneína)

MTP – *metal tolerance protein* (proteína de tolerância à metais)

NA – *nicotianamine*

NAS – *nicotianamine synthase* (nicotianamina sintase)

NRAMP – *natural resistance-associated macrophage protein* (proteína de macrófago associada à resistência natural)

QTL – *quantitative trait locus* (locus de característica quantitative)

RT-PCR – *reverse transcriptase polymerase chain reaction* (transcrição reversa e reação em cadeia da polymerase)

T-DNA – *transfer DNA* (DNA de transferência)

TMD – *transmembrane domain* (domínio transmembrana)

VIT – *vacuolar iron transporter* (transportador vacuolar de ferro)

wt – *wild type* (tipo selvagem)

YS – *yellow stripe*

YSL – *yellow stripe-like* (semelhante *yellow stripe*)

ZAT – *zinc transporter of Arabidopsis thaliana* (trnsportador de zinco de *Arabidopsis thaliana*)

ZIF – *Zinc Induced Facilitator* (facilitador induzido por zinco)

ZIFL – *Zinc Induced Facilitator-Like* (semelhante ao facilitador induzido por zinco)

ZIP – *zinc-regulated/iron-regulated transporter* (transportador zinco-regulado/ ferro-regulado)

ZnT – *Zn transporter* (transportador de Zn)

Sumário

Introdução Geral

1. Arroz

O arroz (*Oryza sativa*) é uma das três monoculturas mais importantes no mundo juntamente com milho (*Zea mays*) e trigo (*Triticum aestivum*). Considerando-se área plantada, o arroz aparece em terceiro, com 159.416.542 hectares; e em segundo quando considerada a produção, com 696.324.394 toneladas (FAO – Food and Agriculture Organization; http://faostat.fao.org).

O Brasil é o nono maior produtor de arroz, e o maior fora do continente asiático, seguido pelos Estados Unidos. Em média, são consumidos 95 g/pessoa/dia no país, colocando o país como o 55º maior consumidor (FAO - http://faostat.fao.org). Cerca de 60% da produção brasileira provém do Rio Grande do Sul (IBGE, 2012), onde diversos municípios baseiam sua economia na produção orizícola, principalmente na metade sul do estado.

O arroz também é considerado uma planta-modelo para estudos em monocotiledôneas. Possui o menor genoma entre as plantas cultivadas, entre 398 a 466 Mb na região eucromática (Goff *et al.*, 2002; Yu *et al.*, 2002; IRGSP, 2005), cerca de 3,7 vezes maior que o genoma da planta-modelo *Arabidopsis thaliana* (*Arabidopsis Genome Initiative* 2000). Adicionalmente, estão completamente sequenciados os genomas das variedades *indica* e *japonica* (IRGSP, 2005; Yu *et al.*, 2002).

Apesar de sua importância para a dieta, o grão de arroz é pobre em nutrientes como ferro (Fe) e zinco (Zn), sendo o cereal com as concentrações mais baixas, e que apresenta a variabilidade genética mais restrita para melhoramento convencional desta característica (Kennedy & Burlingame 2003, Pfeiffer *et al.*, 2008). As deficiências de Fe e Zn em humanos afetam cerca de 30% da população mundial, sendo as duas deficiências minerais com maior prevalência (Gómez-Galera, 2010). Dietas baseadas principalmente em cereais, comuns em países e populações pobres, aumentam os riscos de desnutrição, indicando que esforços para a melhoria da qualidade nutricional de grãos são necessários. A biofortificação surgiu, nos últimos anos, como a solução para aliviar esses problemas de má nutrição. Biofortificação é o processo que consiste em aumentar o conteúdo natural de nutrientes biodisponíveis em plantas cultivadas. As plantas estão no início da cadeia alimentar, portanto, o aperfeiçoamento da retirada de minerais do solo e aumento da sua movimentação e biodisponibilidade nas partes comestíveis fornecem benefícios para nutrição animal e humana (Palmgren *et al.*, 2008).

2. Homeostase de Zn em plantas

Grande parte dos metais, em forma de íons, são encontrados em sistemas vivos possuíndo papéis catalíticos e estruturais em funções biológicas. O Zn é o segundo metal de

transição mais abundante nos organismos (o primeiro é o Fe), apresentando várias características únicas na sua química (Broadley *et al.*, 2007). O Zn é um ácido forte de Lewis que exibe alta afinidade de ligação a bases "macias" (ligantes sulfeto) e bases "duras" (ligantes amino, carboxilato e hidroxil). O Zn^{+2} ocorre num estado único de oxidação e não catalisa a formação de radicais livres. A forma de coordenação geométrica mais comum do Zn^{+2} é a tetraédrica, a qual geralmente representa o complexo ótimo de Zn em cavidades de proteínas, como os dedos de proteína "*zinc fingers*" e enzimas (Fraústo da Silva & Williams, 2001).

Íons metais que são inertes a reações redox, como o Zn, são componentes estruturais chave para um grande número de proteínas. Foi demonstrado que 47% das proteínas estruturais conhecidas necessitam de metais, com 41% contendo metais como seu centro catalítico (Andreini *et al.*, 2008). As propriedades do Zn, como um ácido de Lewis, estabiliza as cargas negativas na conformação de proteínas dobradas, facilitando a interação da proteína com outras macromoléculas, incluindo o ácido desoxirribonucleico (ADN; Berg & Shi, 1996). Recentemente, foi sugerida uma nova atuação para o Zn, em sistemas biológicos, que foi denominada "*matchmaker*", onde este metal ajudaria na interação de domínios únicos de proteína sem participar do produto final (Lu, 2010).

Sobretudo, o Zn é importante na atividade enzimática e regulação gênica por sua presença em fatores de transcrição, sendo descrito como cofator em mais de 300 proteínas (Palmgren *et al.*, 2008). Esse metal também pode atuar como molécula sinal em animais, de forma similar com as rotas dependentes de cálcio (Hirano *et al.*, 2008), entretanto, esta função não foi ainda descrita em plantas.

Todos os organismos possuem um ajuste fino nas redes de homeostase de metais para manter a concentração de metais dentro dos limites fisiológicos. Diferentes espécies de plantas e até variedades da mesma espécie diferem na sua "eficiência de Zn", como, por exemplo, a habilidade de manter o crescimento e o rendimento em condições limitantes de Zn (Graham & Rengel, 1993).

Excesso de Zn pode ser tóxico para as células, provavelmente devido à competição com outros íons biologicamente ativos por sítios de ligação (Clemens, 2001). Sintomas fisiológicos do excesso de Zn em plantas são geralmente clorose das folhas mais jovens, biomassa reduzida e inibição do crescimento da raiz (Broadley *et al.*, 2007). A clorose das folhas pela toxicidade de Zn pode ser relacionada à reduzida captação de Fe decorrente do aumento da concentração de Zn no meio externo (Ren *et al.*, 1993). Os mecanismos de tolerância ao excesso de Zn em plantas incluem exclusão, transporte para órgãos e tecidos específicos, transporte para

compartimentos celulares ou subcelulares, ou sequestro por ligantes específicos (Clemens, 2001).

A deficiência de Zn em plantas é associada com a interrupção da atividade enzimática normal; um problema comum é a inibição da fotossíntese como resultado da redução da atividade de enzimas fotossintéticas chave (Hu *et al.,* 1991). Nos estágios iniciais da deficiência de Zn, as folhas jovens se tornam amareladas e até necróticas. Plantas com severa deficência de Zn parecem atrofiadas e exibem reduzido alongamento (Marschner, 1995). Quando em deficiência de Zn as plantas regulam os transportadores deste metal para aumentar a captação de Zn (Assunção *et al.,* 2010), que pode ser adquirido do solo primariamente como Zn^{+2}, mas também complexado a ligantes orgânicos (Krämer & Clemens, 2005). Depois da captação, o Zn deve ser translocado pela planta e distribuído nos órgãos, tecidos e células para função adequada.

Por ser necessário mas também tóxico, dependendo da concentração, a distribuição e compartimentalização do Zn nos sistemas biológicos é extremamente importante. Em *Escherichia coli*, a quantidade de Zn^{2+} livre foi estimado em menos de um cátion por célula (Outten & O'Halloran, 2001). Com base nessa informação, duas hipóteses podem ser feitas: praticamente nenhum Zn livre é esperado dentro das células; e tanto a distribuição correta entre os diferentes compartimentos subcelulares e dentro da planta, quanto a quelação do Zn são necessários. Para que essas hipóteses sejam possíveis algumas famílias de proteínas, assim como moléculas de ligação a metais, têm sido relacionadas à homeostase de Zn.

A absorção de Zn pelas plantas é pouco conhecida, ocorrendo através de transportadores não-específicos (Palmgren *et al.,* 2008), além de outros mecanismos ainda não identificados. Alguns dos transportadores de Zn já caracterizados incluem AtIRT1, membro da família ZIP (*Zinc-Regulated Transporter (ZRT), Iron-Regulated Transporter (IRT)-Like Protein*) em Arabidopsis, o qual apresenta afinidade por Zn, além de manganês (Mn), cobalto (Co) e cobre (Cu) (Korshunova *et al.,*1999). As proteínas de Arabidopsis AtZIP1, AtZIP2, AtZIP3, AtZIP4 também são capazes de transportar Zn (Grotz & Guerinot, 2006; Milner *et al.,* 2013). Em arroz, OsZIP5, OsZIP4 e OsZIP8 são transportadores exclusivos de Zn (Ishimaru *et al.,* 2007; Yang *et al.,* 2009; Lee *et al.,* 2010a), enquanto OsZIP7 transporta somente Fe (Yang *et al.,* 2009). Da mesma forma, transportadores da família *Yellow-stripe* (YS) apresentam afinidade por Zn (Schaaf *et al.,* 2005), indicando que podem participar na absorção ou na translocação do metal.

A proteína AtHMA4, pertencente à subfamília P_{1B} de ATPases do tipo P, localizada na membrana plasmática (Verret *et al.,* 2005), foi relacionada à homeostase de Zn em *Arabidopsis*

thaliana, participando do processo de translocação de Zn da raiz para parte aérea. Plantas de *Arabidopsi*s mutantes para o gene *HMA4* possuem alta sensibilidade a elevados níveis de Cd e Zn, sugerindo que esta proteína está envolvida na detoxificação de metais (Williams *et al.*, 2005). HMA4 também é responsável pelo transporte de cádmio (Cd; tóxico) a longa distância (Wong & Cobbett, 2009). Outro grupo envolvido na homeostase de metais e expresso em maiores níveis em espécies hiperacumuladoras de Zn são os genes *NAS* que codificam nicotianamina sintases (NASs). A nicotianamina (NA) é um importante quelante de Zn^{+2} nas células (Becher *et al.*, 2004; Weber *et al.*, 2004; van de Mortel *et al.*, 2006).

O vacúolo tem sido explorado como um importante sítio intracelular para acúmulo de metais. Estudos analisando espécies vegetais para uso em fitorremediação vêm desvendando o papel do mesmo na fisiologia de plantas hiperacumuladoras de metais, como *Thlaspi caerulescens, Thlaspi goesingense* e *Arabidopsis halleri* (Hanikenne *et al.*, 2008; Gustin *et al.*, 2009; Oomen *et al.*, 2009). Estas plantas são tolerantes a quantidades até cem vezes maiores de Zn, Cd, níquel (Ni) e outros metais pesados, quando comparadas a espécies não hiperacumuladoras, sendo capazes de extraí-los do solo e acumulá-los nas raízes e folhas sem apresentarem sinais de toxidez (Dräger *et al.*, 2004). Para isso, fazem uso principalmente de transportadores metal-específicos do tonoplasto, os quais retiram quantidades excessivas de metais presentes do citoplasma, acumulando-os no vacúolo de forma não prejudicial (Dräger *et al.*, 2004). Estudos evolutivos comparando espécies próximas, porém contrastantes quanto à capacidade de tolerar altas concentrações de metais, demonstraram que duplicações no genoma que aumentaram o número de cópias de transportadores de metais estão associadas com a maior capacidade de acumulá-los (Dräget *et al.*, 2004; Hanikenne *et al.*, 2008).

Outros trabalhos têm demonstrado que os transportadores vacuolares podem ser importantes na alocação de metais para as sementes. A proteína VIT1, em sementes de Arabidopsis, é importante para o armazenamento de Fe em vacúolos (Kim *et al.*, 2006), sugerindo que a super-expressão desse gene e o consequente aumento do conteúdo de Fe no vacúolo podem ser úteis no desenvolvimento de linhagens biofortificadas. Palmgren et al (2008) sugeriram que a remobilização de Zn vacuolar em cereais durante a senescência pode ser importante para o acúmulo de metais no grão. Outro trabalho demonstrou que genes que codificam transportadores vacuolares de Fe (AtNRAMP3, *natural resistance associated macrphage protein*, Lanquar *et al.*, 2005) e de Zn (MTP, *metal tolerance protein*, Desbrosses-Fonrouge *et al.*, 2005 e ZIF, *zinc induced facilitator*, Haydon & Cobbett, 2007) co-localizam-se com os intervalos de confiança de QTLs (*quantitative trait loci*) que afetam as concentrações de metais em sementes de *Arabidopsis* (Waters & Grusak, 2008). Proteínas

vacuolares também são importantes para a alocação de Zn no grão de cevada, sendo detectada a expressão de proteínas ortólogas a VIT, MTP, NRAMP e ZIF nos tecidos da semente (Tauris *et al.*, 2009).

3. Homeostase de Mn em plantas

O Mn é o segundo metal de transição mais prevalente (depois do Fe) na crosta terrestre sendo um micronutriente essencial para todos os organismos; o Mn possui papel catalisador em reações de transferência de elétrons quando usado como cofator em proteínas (Marschner, 1995). Em comum com outros metais de transição, como Fe e Cu, o Mn pode existir em vários estados de oxidação e disso depende a disponibilidade de Mn para as plantas, pois os estados oxidados Mn^{+3} e Mn^{+5} não são biodisponíveis e acumulados (Clarkson, 1988; Rengel, 2000). É a forma reduzida Mn^{+2} que é transportada para dentro das células vegetais (Laurie *et al.*, 1995). Este metal é de particular importância para organismos fotossintéticos, onde um cluster de átomos de Mn é usado como centro catalítico para oxidação da água, induzida por luz, no fotossistema II; é também usado como cofator numa variedade de enzimas como a superóxido dismutase dependente de Mn (MnSOD; Marschner, 1995).

Apesar de sua importância, a quantidade de Mn exigida pelas plantas é relativamente baixa (Clarkson, 1988). A deficiência de Mn em plantas ocorre em solos calcáreos ou alcalinos que favorecem a oxidação de Mn e imobilização de Mn^{+2}. Ainda quando as plantas estão em ambientes com excesso de Fe, este pode competir com Mn gerando deficiência do mesmo. Os sintomas da deficiência de Mn são denotados por amarelamento de folhas jovens em dicotiledôneas e desenvolvimento de manchas acinzentadas em folhas maduras de cereais (Marschner, 1995).

A alta concentração de Mn causa clorose, manchas marrons e necrose em folhas maduras, resultando em reduzido rendimento da cultura (Marschner, 1995). Esses sintomas parecem resultar da inibição da síntese de clorofila, acúmulo de Mn e polifenóis na parede celular e interferência na homeostase de cálcio, Ca^{+2} (Marschner, 1995). Entretanto, os sintomas da toxicidade de Mn variam amplamente entre as espécies de plantas, assim como as concentrações críticas de Mn em que esses sintomas são manifestados (Bergmann, 1992; Peiter *et al.*,2000; El-Jaoual & Cox, 1998).

Plantas que toleram altas concentrações de Mn podem exibir distintos padrões de compartimentalização, como acúmulo do metal na camada celular epidérmica (González & Lynch, 1999) e deposição nos tricomas (Blamey *et al.*, 1986). Em nível celular, as plantas acumulam altas concentrações de Mn em vacúolos e em algum nível em cloroplastos

(Quiquampoix *et al.*, 1993); a expressão ectópica de transportadores vacuolares de Mn pode aumentar a tolerância das plantas a este metal (Hirschi *et al.*, 2000; Delhaize *et al.*, 2003). Adicionalmente, já foi demonstrado que Mn pode ser acumulado no retículo endoplasmático (Wu *et al.*, 2002). Dessa maneira, o Mn pode ser exportado para outras endomembranas além do vacúolo, embora seu acúmulo em outras organelas seja menor do que no vacúolo.

O mecanismo de transporte e homeostase de Mn^{+2} é bem caracterizado em microrganismos; em muitas espécies de bactérias e em levedura (*Saccharomyces cerevisiae*) várias rotas de transporte de Mn já foram identificadas (Jakubovics & Jenkinson, 2001; Luk *et al.*, 2003). Foi observado que muitos desses transportadores possuem ampla especificidade de substrato, o que também é esperado em plantas. Alguns membros da família de transportadores *ZIP* de *Medicago truncatula* e *Solanum lycopersicum*, e AtIRT1 de Arabidopsis são capazes de transportar Mn^{+2} além de outros metais (López-Millán *et al.*, 2004; Eckhardt *et al.*, 2001; Korshunova *et al.*, 1999). OsYSL2 de arroz pode transportar Mn^{+2} e Fe^{+2} quelados a nicotianamina (NA) na mesma proporção através da membrana plasmática para internalização e distribuição dentro do floema para transporte de longa distância (Koike *et al.*, 2004).

Intracelularmente a Ca^{+2} ATPase ECA1 parece fornecer Mn para o retículo endoplasmático em plantas (Wu *et al.*, 2002). No vacúolo, AtCAX2 (um membro da família transportadora de Ca^{+2} em Arabidopsis) e ShMTP8 (membro da família CDF de *Stylosanthes hamata*) foram caracterizados como transportadores de Mn (Pittman *et al.*, 2004; Schaaf *et al.*, 2002; Gustin *et al.*, 2011). AtMTP11, membro da família CDF de Arabidopsis, possui um papel determinante na tolerância ao excesso de Mn. Localizado num compartimento associado ao golgi, este transportador deve estar envolvido num mecanismo de tolerância envolvendo tráfego vesicular e exocitose (Peiter *et al.*, 2007; Delhaize *et al.*, 2007).

4. A família de proteínas facilitadora de difusão de cátions (CDF)

Tendo em vista o foco deste trabalho, vamos dedicar uma seção desta introdução para um detalhamento desta família de transportadores de metais e seus integrantes já caracterizados na literatura. As CDFs são proteínas integrais de membranas que ou transportam cátions divalentes do citoplasma para o espaço extracellular, ou para dentro de compartimentos internos como o vacúolo. É conhecido apenas um caso de proteína CDF que transporta Zn para o citoplasma (Cragg *et al.*, 2002). As proteínas CDF atuam como transportadores do tipo antiporter de metais divalentes e prótons (Me^{+2}/H^{+}) (Guffanti *et al.*, 2002; Chao *et al.*, 2004; Grass *et al.*, 2005; Kawachi *et al.*, 2008), possuem porções citoplasmáticas N e C terminais e geralmente seis domínios transmembrana (TMD; Paulsen & Saier,1997).

As CDFs são encontradas em bactérias, archae e em eucariotos (Montanini *et al.,* 2007). Com base na análise filogenética e especificidade de substrato de proteínas representativas, as CDFs são agrupadas em Zn-CDFs, Zn/Fe-CDFs e Mn-CDFs. As CDFs de plantas são chamadas de proteína de tolerância a metais (MTP), enquanto os membros de vertebrados são chamados transportadores de Zn (ZnT) ou família carreadora de soluto 30 (SLC30) (Montanini *et al.,* 2007).

A principal família de transportadores reconhecidamente envolvida na captação de Zn é a família ZIP (Zhao *et al.,* 1996a; Zhao *et al.,* 1996b; Grotz *et al.,* 1998; Gaither *et al.,* 2000). Esse conhecimento é baseado em levedura, onde as proteínas de alta afinidade de membrana plasmática, ZRT1 e ZRT2, são reguladas positivamente por baixas concentrações de Zn, enquanto modificações pós-traducionais as "retiram" da membrana plasmática, após reabastecimento de Zn (Gitan *et al.,* 1998). Mecanismos similares são observados em sistemas de mamíferos (Kim *et al.,* 2003; Wang *et al.,* 2004). Entretanto, mudando as condições de baixa concentração de Zn para concentracões suficientes deste metal, causa-se um "choque de Zn", ou seja, um alto influxo de Zn no citoplasma causado por ZRT1 e ZRT2 antes de suas remoções da membrana (MacDiarmid *et al.,* 2003). Duas proteínas, ZRC1 e COT1, são necessárias para detoxificação do Zn em excesso nessa condição, como também em condições de concentrações tóxicas de Zn (Kamizono *et al.,* 1989; Conklin *et al.,* 1992; Conklin *et al.,* 1994; MacDiarmid *et al.,* 2003). Essas proteínas são membros da família CDF (Gaither & Eide, 2001), e foi demonstrado que transportam Zn para dentro do vacúolo (MacDiarmid *et al.,* 2003; Conklin *et al.,* 1994).

Em plantas, filogenias adicionais dividem as proteínas CDF em sete grupos distintos, o nome dos grupos segue a nomenclatura das sequências de MTP de *Arabidospis thaliana*. Grupos 1, 12 e 5 são parte das Zn-CDFs; grupos 5 e 6 das Fe/Zn-CDFs; e grupos 8 e 9 das Mn-CDFs (Gustin *et al.,* 2011). Até o momento, as únicas proteínas CDF caracterizadas são membros do grupo 1, que inclui os transportadores vacuolares de Zn AtMTP1 e AtMTP3 (Desbrosses-Fonrouge *et al.,* 2005; Kobae *et al.,* 2004; Kawachi *et al.,* 2009); e do grupo 8 e 9, o qual inclui o transportador localizado em compartimentos pré-vacuolares/trans golgi AtMTP11 (Peiter *et al.,* 2007; Delhaize *et al.,* 2007). Existem 12 membros da família CDF no genoma de *Arabidopsis thaliana*, e dez em *Oryza sativa* (Gustin *et al.,* 2011).

4.1 CDF em plantas - grupo 1

A primeira proteína CDF descrita em plantas foi denominada ZAT (transportador de Zn) em *Arabidopsis thaliana*, renomeada mais tarde como AtMTP1 (metal tolerance protein; van der Zaal *et al.*, 1999; Maser *et al.*, 2001; Delhaize *et al.*, 2003). A atividade de MTP1 no transporte de Zn foi pela primeira vez demonstrada em proteolipossomos reconstituídos de *Escherichia coli* (Bloss *et al.*, 2002) e então pela complementação do duplo-mutante de levedura *zrc1 cot1*, o qual é altamente sensível ao excesso de Zn (Drager *et al.*, 2004; Kim *et al.*, 2004).

O nível de transcritos de *AtMTP1* é mais alto em raízes do que em parte aérea, não é afetado pelo status de Zn, e MTP1 fusionado a GFP foi localizado na membrana vacuolar (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; Kawachi *et al.*, 2009). A atividade do promotor de *AtMTP1* é mais proeminente em folhas jovens e raízes, diminuindo em plantas velhas, mas ainda detectada na ponta da raiz principal e em raízes laterais. Também foi detectada em botões florais, principalmente em carpelos e grãos de pólen, como também no embrião e no endosperma (Desbrosses-Fonrouge *et al.*, 2005). Outro trabalho descreveu um padrão de atividade similar do promotor, exceto que pêlos radiculares e células do estômato também mostraram expressão (Kawachi *et al.*, 2009).

Plantas de Arabidopsis mutantes para *MTP1* ou contendo constructos de RNAi para *MTP1* possuem maior sensibilidade a Zn do que plantas wt, enquanto a superexpressão de *MTP1* aumenta a resistência a excesso de Zn (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; van der Zaal *et al.*, 1999). Comparando secções transversais de folhas de mutates *atmtp1* e plantas wt, foi observado que as folhas de *atmtp1* mostram plasmólise celular, dano no parênquima e perda de células do mesófilo quando em excesso de Zn (Kobae *et al.*, 2004).

AtMTP3 é outro membro bem caracterizado da família gênica MTP em *Arabidopsis thaliana*. A proteína é um transportador vacuolar de Zn expresso principalmente em células epidermais e corticais de raízes, tendo seu principal papel na tolerância basal de Zn (Arrivault *et al.*, 2006). A superexpressão de *AtMTP3* aumenta o acúmulo de Zn em raízes e folhas, e aumenta também a tolerância. Ao contrário, quando silenciado por RNAi, as plantas se tornam mais sensíveis a Zn, e acumulam maiores concentrações de Zn na parte aérea (Arrivault *et al.*, 2006). Interessantemente, os transcritos de *AtMTP3* são regulados por excesso de Zn, Co e Mn, e também na deficiência de Fe. O padrão de atividade do promotor de *AtMTP3* é similar ao de *AtFRO2* (Fe redutase de membrana plasmática) e *AtIRT1* (transportador de Fe^{+2} na membrana plasmática), que são necessários para captação de Fe nas raízes (Eide *et al.*, 1996; Connolly *et al.*, 2002; Vert *et al.*, 2002).

Como AtIRT1 tem uma grande quantidade de substratos (Korshunova *et al.,* 1999; Eide *et al.,* 1996), a deficiência de Fe induz um alto influxo de Zn nas raízes, o qual pode ser detoxificado pela entrada para dentro do vacúolo, realizada pelo transportador AtMTP3. Interessantemente, a regulação do excesso de Zn e da deficiência de Fe parecem ser independentes (Arrivault *et al.,* 2006). A exclusão de Zn nas raízes é um mecanismo importante para a tolerância basal de Zn (Becher *et al.,* 2004; Weber *et al.,* 2004). O silenciamento de *AtMTP3* ou *AtMTP1* (ou mutante de T-DNA de *atmtp1*; Desbrosses-Fonrouge *et al.,* 2005; Kobae *et al.,* 2004; Kawachi *et al.,* 2009), ambos expressos em raízes, levam a sensibilidade a Zn, o que sugere que eles não conseguem compensar um ao outro e, portanto, não possuem funções redundantes na tolerância a Zn (Arrivault *et al.,* 2006).

A atividade do promotor de *AtMTP3* se sobrepõe apenas parcialmente com *AtMTP1* em raízes, visto que o primeiro tem expressão baixa, mas induzida, localizada na epiderme e no córtex dos pelos radiculares, enquanto o segundo possui expressão constitutiva, localizada no meristema e nas zonas de alongamento. Na parte aérea, a atividade do promotor de *AtMTP3* é indetectável, enquanto *AtMTP1* é expresso em folhas jovens (Desbrosses-Fonrouge *et al.,* 2005; Arrivault *et al.,* 2006). As concentrações de Zn em plântulas de *atmtp1* são baixas nas raízes em condições de alta e baixa concentração de Zn, quando comparadas com wt, enquanto a concentração na parte aérea não difere entre wt e *atmtp1* (Kawachi *et al.,* 2009). Esses resultados sugerem que AtMTP1 é essencial para a detoxificação de Zn, presumivelmente pela exclusão para dentro do vacúolo (Kawachi *et al.,* 2009).

Foi proposto que a função de AtMTP1 é de sequestrar Zn em tecidos sensíveis, em divisão e expansão, gerando o armazenamento de Zn em tecidos específicos da parte aérea, enquanto AtMTP3 deve estar envolvido na remoção de Zn da rota de translocação da raiz para parte aérea sob alto influxo de Zn (Sinclair & Kramer, 2012). Consistente com esta função, foi demonstrado que os níveis de expressão de *AtMTP1* em ecótipos distintos de *Arabidopsis thaliana* são correlacionados com o acúmulo de Zn nas folhas (Conn *et al.,* 2012).

Resultados similares foram observados para o órtologo de *AtMTP1, PtdMTP1,* do álamo híbrido (*Populus trichocarpa* X *Populus deltoides*). *PtdMTP1* é constitutivamente expresso, complementa o mutante de levedura *zrc1* e o mutante *cot1* e se localiza na membrana vacuolar (Blaudez *et al.,* 2003). Quando superexpresso em Arabidopsis, *PtdMTP1* confere elevada tolerância a Zn.

Em arroz, *OsMTP1* foi recentemente descrito como sendo induzido por Zn, Cd, Cu, magnésio (Mg) e Fe, (Yuan *et al.,* 2012; Lan *et al.,* 2012). Plantas de arroz silenciadas para *OsMTP1* demonstraram menor tolerância a Zn, Cd e Ni. A expressão de *OsMTP1* em levedura

selvagem BY4741, durante a fase de crescimento exponencial, aumentou a tolerância a Zn, Cd e Ni, porém, OsMTP1 não foi capaz de complementar o mutante de levedura *pmr1* sensível a Mn (Yuan *et al.,* 2012). A localização da proteína OsMTP1 em membrana plasmática foi sugerida quando fusionada com GFP e transientemente expressa em células epidérmicas de cebola (Yuan *et al.,* 2012). Também foi sugerida a localização de OsMTP1 na membrana vacuolar quando fusionada com GFP e expressa em *Saccharomyces cerevisiae* (Lan *et al.,* 2012). A localização de OsMTP1 na membrna plasmática é pouco provável comparando a localização das proteínas MTP1 de outras espécies de plantas já caracterizadas (Gustin *et al.,*2009; Kobae *et al.,* 2004; Drager *et al.,* 2004; Desbrosses-Fonrouge *et al.,* 2005; Arrivault *et al.,* 2006; Blaudez *et al.,* 2003).

4.2 CDF em plantas - grupo 8 e 9

No grupo 9, a primeira proteína caracterizada foi ShMTP8 (originalmente chamada ShMTP1, teve seu nome modificado para manter a consistência com a nomenclatura de Arabidopsis), presente na leguminosa tropical *Stylosanthes hamata*, hiperacumuladora de Mn, confere tolerância a Mn em levedura selvagem através do sequestro de Mn^{2+} a uma organela interna, ao invés de causar o efluxo de Mn^{2+} para o meio externo (Delhaize *et al.,* 2003). Este transporte de Mn deve funcionar como um sistema antiporte H^{+}:Mn^{2+}, devido ao requerimento de uma H^{+}ATPase tipo-V ativa para a efetiva tolerância a Mn. Em plantas (Arabidopsis e tabaco), a superexpressão de *ShMTP8* também conferiu tolerância a Mn; a proteína fusionada a GFP foi localizada especificamente no tonoplasto, sugerindo fortemente que o sequestro de Mn^{2+} no vacúolo é o mecanismo conferindo tolerância a este metal (Delahaize *et al.,* 2003).

A alta similaridade de ShMTP8 com AtMTP8 a AtMTP11 sugere que essas proteínas possuem funções similares em plantas. Dos quarto genes testados, *AtMTP11* demonstrou ter os mais altos níveis de expressão em plântulas de Arabidopsis crescidas em diferentes concentrações de Mn^{2+}. Como esperado, quando expresso em levedura, *AtMTP11* conferiu tolerância a Mn^{2+} e Cu^{2+} (em menor nível), mas não aumentou a tolerância a uma série de outros metais, como Zn^{2+}, Co^{2+} e Ni^{2+}. Usando vesículas de membrana microssomais de levedura, foi demonstrado que AtMTP11 confere atividade transportadora de prótons dependente de Mn^{2+} (Delhaize *et al.,* 2007). Diferentemente de ShMTP8, a proteína AtMTP11 não foi localizada na membrana vacuolar, mas sim em compartimentos pré-vacuolares do trans-golgi (Delhaize *et al.,* 2007; Peiter *et al.,* 2007). Dessa maneira, a tolerância a Mn conferida por AtMTP11 provavelmente depende do tráfego vesicular e exocitose do excesso de Mn^{2+} (Peiter *et al.,* 2007), corroborando com o aumento do acúmulo de Mn em folhas do

mutante *atmtp11* de Arabidopsis. O mesmo mutante é hipersensível a elevados níveis de Mn, enquanto plantas superexpressando são hipertolerantes (Delhaize *et al.,* 2007; Peiter *et al.,* 2007). Interessantemente, dois genes homólogos em álamo, *PtMTP11.1* e *PtMTP11.2*, também codificam proteínas que localizam-se em compartimentos do tipo golgi e são capazes de complementar o mutante *atmtp11* de Arabidopsis, sugerindo que as proteínas MTP11 de álamo e Arabidopsis possuem funções semelhantes (Peiter *et al.,* 2007).

4.3 Reconhecimento e transporte de metais em proteínas CDF

Nas proteínas do grupo Zn-CDF, uma alça de histidina (His) é comumente encontrada entre os domínios transmembrana TMD IV e TMD V. Sugerida como um sítio de ligação para Zn, esta alça pode agir como um sensor das concentrações citoplasmáticas deste metal (Montanini *et al.,* 2007; Kawachi *et al.,* 2008). A remoção da alça de His da proteína TcMTP1 (do hiperacumulador *Thlaspi caerulescens* também conhecido atualmente como *Noccaea caerulescens*) resulta numa proteína não funcional (Kim *et al.,* 2004). Entretanto, a deleção de 32 resíduos da porção N-terminal da alça de AtMTP1 confere a habilidade de complementar o fenótipo de linhagens de levedura mutante sensíveis a Co, enquanto ainda transportando Zn (Kawachi *et al.,* 2008). Além de transportar Zn, a proteína AtMTP3 também transporta Co, diferentemente de AtMTP1 (Arrivault *et al.,* 2006).

Interessantemente, a proteína HvMTP1 de cevada (*Hordeum vulgare*), também localizada no tonoplasto, é capaz de resgatar o fenótipo de leveduras mutantes sensíveis a Zn e Co. Trocando a alça de His de AtMTP1 para HvMTP1, este perde a atividade transportadora de Co. Uma região de cinco resíduos (VTVTT) confere especificidade de transporte para Zn quando presente na alça de His de AtMTP1 (Podar *et al.,* 2012). Resíduos adicionais entre TMD II e TMD III também estão envolvidos na especificidade por Zn; mutações únicas são capazes de conferir atividade de transporte de Co para AtMTP1 (Podar *et al.,* 2012). Mutações similares abolem o transporte de Zn, mas conferem a habilidade de transportar Fe e Mn na proteína ScZRC1 de levedura (Lin *et al.,* 2009), indicando que esta região é importante para seletividade de metais.

Numa comparação entre o transportador de mamíferos Zn-específico ZnTs e o transportador bacteriano YiiP capaz de transportar Zn e Cd, foi recentemente demonstrado que uma mutação de His para ácido aspártico (Asp) no motivo tetraédrico de transporte de metais é responsável pela seletividade de metais (Hoch *et al.,* 2012; Wei & Fu 2005; Lu *et al.,* 2009). Por consequência, a troca desses resíduos entre os genes de mamíferos e bactéria confere atividade de transporte de Cd para proteínas ZnT, enquanto prejudica a mesma atividade no

homólogo de bactéria (Hoch *et al.,* 2012). Como diferentes metais podem ser transportados por proteínas semelhantes, o entendimento de como essas proteínas distinguem cada um dos metais é uma informação chave para aplicações na fitorremediação e/ou biofortificação.

5. Biofortificação

A biofortificação (aumento de micronutrientes em partes comestíveis de culturas alimentares) é a primeira ferramenta sendo empregada na agricultura para tratar a deficiência de micronutrientes em todo o mundo (Bouis & Welch, 2010). Ao contrário da fortificação tradicional de alimentos, a biofortificação não exige o processamento do alimento, uma vez que o micronutriente já está presente nas culturas em crescimento, deixando o "produto" mais acessível para aqueles que consumem alimentos crescidos localmente (HarvestPlus, 2010). Pouca intervenção ou investimento é necessário uma vez que agricultores locais tenham adotado essas novas sementes. A estratégia de biofortificação pode ser alcançada por diferentes abordagens como cruzamentos convencionais e/ou engenharia genética (Sperotto *et al.,* 2012).

5.1 Biofortificação - cruzamentos convencionais

Esforços consideráveis têm sido realizados para alcançar a meta de biofortificação de culturas alimentares através de cruzamentos convencionais (Graham *et al.,* 1999; Cakmak *et al.,* 2000; Toenniessen, 2002; Vasconcelos *et al.,* 2003), mas um melhor entendimento dos processos fisiológicos e moleculares envolvidos neste processo ainda é necessário; este conhecimento poderá facilitar os programas de cruzamento (Clemens *et al.,* 2002).

Alguns critérios são necessários para o uso do cruzamento convencional em programas de biofortificação: a existência de variação genética (isto é, material parental com alto conteúdo de micronutriente no grão), métodos de seleção e marcadores apropriados, e herdabilidades viáveis. Também, as novas variedades estabelecidas devem combinar as características desejadas com alto rendimento, aumentando o custo dos programas de cruzamento consideravelmente, porém, a relação custo-benefício deve ser maior (Graham *et al.,* 1999).

A concentração de Zn em sementes de feijão é uma das maiores entre as fontes vegetais. A avaliação da coleção principal de sementes de feijão do CIAT (Centro Internacional de Agricultura Tropical) revelou uma variação de 21 a 54 mg de Zn por Kg de peso seco (DW) (Tohme *et al.,* 1994). No caso das cultivares de trigo modernas, a variação na concentração de Zn nas sementes é relativamente baixa (Cakmak *et al.,* 2000). Entretanto, como indicado por Cakmak et al (1999), alguns tipos de trigo selvagem são fontes importantes

de material genético para o aumento da concentração de micronutrientes em sementes. De fato, a concentração mais alta de Zn foi encontrada em sementes de trigo selvagem ssp. *boeoticum* (178 mg/Kg DW) e ssp. *dicoccoides* (159 mg/Kg DW), sugerindo que espécies de trigo selvagem podem ser utilizadas como fontes de diversidade genética para o aumento do acúmulo de Zn em sementes de trigo de linhagens modernas (Cakmak *et al.*, 2000).

Entre 939 amostras de arroz integral analisadas, a concentração de Zn variou de 15.9 a 58.4 mg/Kg DW, com um média de 25.4 mg/Kg DW (Graham *et al.*, 1999). O genótipo de arroz IR68144 contém 34 mg Zn/Kg DW; possui um grão de qualidade e alto rendimento; estudos preliminares demonstraram que estas sementes podem melhorar a nutrição humana (Gregorio et al., 2000). Além disso, IR68144 possui baixos níveis do anti-nutriente ácido fítico em grãos polidos, com a maior parte do fitato localizado na fração do farelo (Prom-u-thai *et al.*, 2006). De acordo com Raboy (2006), o baixo nível de ácido fítico pode resultar em uma nutrição aprimorada através do aumento da biodisponibilidade de Zn já existente nos grãos dos cereais.

Diversos mutantes com baixo nível de ácido fítico (*lpa*) têm sido utilizados em programas de melhoramento, após testes em condições de campo (Raboy, 2002; Liu *et al.*, 2007). Lönnerdal et al (2011) demonstraram que genótipos mutantes (*lpa*) de milho, arroz e cevada aumentam a absorção de Zn no modelo de amamentação de filhotes de rato. Em humanos, estudos nutricionais demonstraram que a absorção de Zn de refeições preparadas com milho mutante *lpa* é mais alta do que de refeições preparadas com milho selvagem (Hambidge *et al.*, 2004). Entretanto, Welch (2002) sugere que o baixo nível de fitato em plantas pode ser prejudicial em sistemas de melhoramento, já que mutantes *lpa* são geralmente associados com efeitos negativos na fisiologia da semente e na "performance" da planta (Bregitzer & Raboy, 2006).

5.2 Biofortificação – engenharia genética

A engenharia genética é uma ferramenta promissora para o aumento da biodisponibilidade de Zn em culturas importantes para a alimentação humana, pois o limite da melhoria no acúmulo de Zn por cruzamentos convencionais é dado pela diversidade na variabilidade genética dentro de coleções de germoplasmas existentes. Portanto, abordagens genéticas são necessárias para aumentar significativamente o acúmulo de Zn nos grãos. Entretantato, nenhuma das estratégias testadas até o momento foi totalmente bem sucedida. São consideradas cinco possíveis etapas limitantes para o processo de biofortificação: absorção nas

raízes; carregamento no xilema; remobilização de metais das folhas e carregamento do floema; descarregamento no grãos; e força de dreno do grãos.

As concentrações “alvo” de Zn estabelecidas pelo programa HarvestPlus são 28 mg/Kg DW em arroz polido, 38 mg/Kg DW em grãos de trigo, 38 mg/Kg DW em milho e 56 mg/Kg DW em feijão (Bouis & Welch, 2010). Diversos trabalhos observaram alterações nas concentrações de Fe e Zn quando genes foram mutados ou super-expressos, porém poucos tiveram resultados com potencial para a biofortificação.

A manipulação da expressão de transportadores ZIP foi investigada como uma estratégia para biofortificação em muitas espécies. A superexpressão de *OsZIP4* em plantas de arroz resulta em baixos níveis de Zn nas sementes (Ishimaru *et al.,* 2007). Interessantemente, essas plantas transgênicas mostraram maior concentração de Fe na parte aérea (1.5 vezes) quando comparadas com wt. Considerando que *OsZIP4* não transporta Fe em testes de complementação em levedura (Ishimaru *et al.,* 2005), esses resultados apoiam a idéia de que mudanças na homeostase de um único metal geralmente alteram a homeostase de outros metais no sistema (Spertotto *et al.,* 2012). Efeitos similares na concentração de Zn em sementes foram observados com a superexpressão de *OsZIP5* (Lee *et al.,* 2010b) e *OsZIP8* (Lee *et al.,* 2010a). Por outro lado, a superexpressão de *AtZIP1* em cevada aumentou a concentração de Zn no grão em torno de 2.7 vezes (Ramesh *et al.,* 2004).

A rota de biossíntese de NA também tem sido explorada. Grãos polidos de arroz transgênico expressando o gene *HvNAS1* de cevada sob o controle do promotor de arroz actina1 contém 2.5 vezes mais zinco do que wt (Masuda *et al.,* 2009). A superexpressão do mesmo gene *HvNAS1* em plantas de tabaco aumentou 1.8 vezes a concentração de Zn nas sementes (Takahashi *et al.,* 2003). A ativação por *activation-tagging* dos genes *OsNAS2* e *OsNAS3* causou aumentos de cerca de 2 vezes nas concentrações de Fe e Zn. Testes demostraram que, quando alimentados com o arroz transgênico, camundongos absorveram maiores quantidades desses metais (Lee *et al.,* 2009, Lee *et al.,* 2011). A superexpressão de genes NAS tem se mostrado uma boa estratégia para biofortificação: no melhor resultado descrito na literatura até o momento, Johnson et al (2011) mostraram aumentos de 2 e 4 vezes nas concentrações de Zn e Fe, respectivamente, quando o gene *OsNAS2* foi super-expresso em plantas de arroz. Neste mesmo trabalho, as sementes foram analisadas por *synchrotron X-rayfluorescence spectroscopy* demonstrando que as superexpressão de *OsNAS2* leva ao enriquecimento de Fe e Zn em regiões do endosperma livre de fósforo. No entanto, nenhum teste de biodisponibilidade para absorção foi conduzido com essas plantas, assim como não se tem dados sobre a estabilidade da construção e do fenótipo após múltiplas gerações (Johnson *et*

al., 2011).

6. Candidatos para futuras estratégias de biofortificação / Zn

Estudos recentes apontam alguns candidatos interessantes para futuros estudos de biofortificação de Zn. O fator de transcrição OsNAC5 foi correlacionado positivamente com a alocação de Zn para grãos de arroz (Sperotto *et al.,* 2009). Em trigo, o gene homólogo *TaNAM-B1* regula a senescência foliar, afetando a remobilização de Zn das folhas para os grãos (Uauy *et al.,* 2006). Embora ainda não existam evidências diretas, o gene *OsNAC5* pode ser funcionalmente similar a TaNAM-B1, fornecendo um candidato interessante para manipular a concentração de Zn nos grãos, como também a concentração de Fe e proteína (Uauy *et al.,* 2006; Sperotto *et al.,* 2009).

A proteína metalotioneína 4 (MT4) possui uma ampla capacidade de ligação a Zn e deve funcionar como uma molécula "armazenadora" deste metal em grãos maduros e em desenvolvimento (Hegelund *et al.,* 2012). A localização de HvMT4 de cevada é limitada ao embrião e camada de aleurona (Hegelund *et al.,* 2012), como também a de AtMTP4a e AtMTP4b de Arabidopsis se limita a embriões tardios e sementes em desenvolvimento (Ren et al., 2012). Isso faz dos *MTs* do tipo 4 interessantes candidatos para futuros trabalhos de biofortificação.

Interessantemente, os transportadores vacuolares de arroz OsVIT1 e OsVIT2 também transportam Zn, ao contrário do seu ortólogo de *Arabidopsis thaliana* AtVIT1 (Kim *et al.,* 2006, Zhang *et al.,* 2012). Assim, plantas mutantes com perda de função em qualquer um dos dois genes VIT de arroz apresentam redução da concentração de Zn nos grãos maduros, uma vez que ambas armazenam Zn (e Fe) nos vacúolos das folhas bandeiras, tornando-o menos disponível para remobilização (Zhang *et al.,* 2012). Além disso, como mutações nos genes VIT causam alteração na localização de Fe no grão, é possível que esses mutantes apresentem diferenças na localização de Zn, embora não tenham sido feitas análises para comprovar essa hipótese (Zhang *et al.,* 2012).

Membros da família gênica de transportadores Zinc-Induced Facilitator-Like (ZIFL) estão envolvidos na homeostase de Zn; *AtZIF1* e *AtZIF1-Like* de Arabidopsis foram descritos como candidatos para aumento da concentração de Zn nas sementes em análises de QTL (Waters & Grusak, 2008). Análises de microarranjo em cevada demonstaram que o gene ZIF1-Like é expresso na camada de aleurona de sementes e sua expressão aumenta no embrião, após fertilização foliar com Zn (Tauris *et al.,*2009).

Objetivos

Objetivo Geral

Identificar e caracterizar novos genes potencialmente envolvidos com a homeostase de Zn e Mn em plantas de arroz, utilizando similaridade de sequência com genes conhecidos, expressão heteróloga em linhagens mutantes de *Saccharomyces cereviseae* e complementação de mutantes de *Arabidopsis thaliana*.

Objetivos específicos

- Identificar sequências similares ao gene *AtMTP1 e AtMTP11* no genoma de arroz;

- Avaliar a atividade transportadora de OsMTP1 e OsMTP11 analisando a importância de resíduos chave na habilidade de transporte e especificidade de ligação a metais em testes de expressão heteróloga em levedura;

- Avaliar a capacidade dos genes de arroz *OsMTP1 e OsMTP11* complementarem os mutantes *mtp1-1 e mtp11* de *Arabidopsis thaliana*, respectivamente;

- Identificar a localização subcelular da proteína OsMTP1 íntegra e com diversas mutações sítio-dirigidas em células de levedura;

- Determinar a localização subcelular da proteína OsMTP1 em plantas homozigotas *mtp1-1* de *A. thaliana* superexpressando *OsMTP1*-GFP.

Capítulo 1

Artigo submetido ao periódico *Journal of Experimental Botany*

(Fator de Impacto JCR 2011 = 5.34)

Functional analysis of the rice vacuolar zinc transporter OsMTP1

Short running title: The vacuolar zinc transporter, OsMTP1

Paloma K. Menguer[1], Emily Farthing[2], Kerry A. Peaston[2], Felipe Klein Ricachenevsky[3], Janette Palma Fett[1,3], Lorraine E. Williams[2]

[1]Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre, 91501-970, Brazil.

[2]Centre for Biological Science, University of Southampton, Life Sciences Building 85,Highfield Campus, Southampton SO17 1B, Hampshire, United Kingdom

[3]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, P.O.Box 15005, Porto Alegre, 91501-970, Brazil.

Paloma Koprovski Menguer paloma.menguer@gmail.com

Emily Farthing ef7g09@soton.ac.uk

Kerry Peaston K.A.Peaston@soton.ac.uk

Felipe Klein Ricachenevsky felipecruzalta@yahoo.com.br

Janette Palma Fett jpfett@cbiot.ufrgs.br

Lorraine E. Williams L.E.Williams@soton.ac.uk Tel: +44(0) 23 80594278 Fax: +44(0) 23 8059 5159

Date of submission: 28.12.2012

Number of figures: 11

Figures in colour in print: 3

Figures in colour online: 3

Number of tables: 2

Word count: 9359

Abstract

Heavy metal homeostasis is maintained in plant cells by specialized transporters, which compartmentalise or efflux metal ions, maintaining cytosolic concentrations within a narrow range. OsMTP1 is a member of the CDF/MTP family of metal cation transporters in *Oryza sativa,* which is closely related to *Arabidopsis thaliana* MTP1. Functional complementation of the Arabidopsis T-DNA insertion mutant *mtp1-1* demonstrates that OsMTP1 transports Zn *in planta* and localises at the tonoplast. When heterologously expressed in the yeast mutant *zrc1 cot1*, *OsMTP1* complemented its Zn-hypersensitivity and was also localised to the vacuole. OsMTP1 alleviated, to some extent, the Co sensitivity of this mutant, rescued the Fe hypersensitivity of the *ccc1* mutant at low Fe concentrations and restored growth of the Cd hypersensitive mutant *ycf1* at low Cd concentrations. These results suggest that OsMTP1 transports Zn but also Co, Fe and Cd, possibly with lower affinity. Site-directed mutagenesis studies revealed two substitutions in OsMTP1, which appear to alter the transport function of OsMTP1. OsMTP1 harbouring a substitution of leucine 82 to a phenylalanine can still transport low levels of Zn, with an enhanced affinity for Fe and Co, and a gain of function for Mn. A substitution of histidine 90 to an aspartic acid completely abolishes Zn transport but improves Fe transport in OsMTP1. The mutated amino acid residues seem to be important in determining substrate specificity and may be a starting point for refining transporter activity in possible biotechnological applications, such as biofortification and phytoremediation.

Keywords: cation diffusion facilitator; ion selectivity; metal tolerance protein; *Oryza sativa*; vacuole; zinc transporter

Abbreviations: CDFs, Cation Diffusion Facilitators; DMF, *N*,*N*-dimethylformamide; EGFP, Enhanced Green Fluorescent Protein; MTPs, Metal Tolerance Proteins; TMDs, Transmembrane Domains.

Introduction

Rice (*Oryza sativa*) is one of the most widely consumed cereals, being cultivated in approximately 156 million hectares, with around 660 million tons harvested in 2008 (IRRI, 2010). Rice is the main carbohydrate source for more than half the world's population. It is mainly consumed in its polished form, which is very poor in micronutrients such as iron (Fe) and zinc (Zn). Human Fe nutritional deficiency affects three billion people, being the most common mineral nutritional disorder, while Zn deficiency is the second (Welch and Graham, 2004). Therefore, it is important to understand the mechanisms of micronutrient import to the rice grain and develop strategies to increase the levels of these nutrients (Wirth *et al.*, 2009). Zn is important for enzymatic activity and for gene regulation by transcription factors, being described as a cofactor in more than 300 proteins (Palmgren *et al.*, 2008). Zn is essential for completing a plant's lifecycle, but is toxic above threshold concentrations. Therefore, it must be maintained within a narrow adequate range. This is regulated in plant cells by specialized transporters, controlling the distribution of metal ions within the cell. Those at the plasma membrane transport ions into or out of the cell. Others, positioned at subcellular membranes, compartmentalize ions when concentrations within the cellular fluid become toxic; this often involves transport to the vacuole.

An array of transporter families exists in plants allowing them to cope with varying substrate availabilities, enabling responses to a variety of stress conditions and meeting specific transport requirements (Hall and Williams, 2003). Cation Diffusion Facilitators (CDFs) or Metal Tolerance Proteins (MTPs) (Nies and Silver, 1995) are ubiquitous in all branches of life (Montanini *et al.*, 20007; Gustin *et al.*, 2011). Structural analysis suggests most family members possess six transmembrane domains (TMDs), with cytoplasmic N- and C-termini (Paulsen and Saier, 1997). Key polar and charged residues conserved within TMDs I, II, V and VI are likely involved in metal transport (Gaither and Eide, 2001; Haney *et al.*, 2005). They contain a histidine-rich cytoplasmic loop, thought to be vital for transporter specificity, which might act as a chaperone determining the identity of metal ions to be transported (Kawachi *et al.*, 2008; Kawachi *et al.*, 2012; Podar *et al.*, 2012). A signature sequence, proposed by Paulsen and Saier (1997) and modified by Montanini et al (2007), enables predictions regarding uncharacterized MTP family members. Members of this family cluster phylogenetically according to their main substrate: Zn, Zn and Fe, or manganese (Mn). It has been suggested that metal selectivity of uncharacterized members can be inferred according to their cluster position (Montanini *et al.*, 2007).

Twelve *MTP* genes have been classified in Arabidopsis and ten in rice (Gustin *et al.,* 2011). The first, identified as *ZAT* or Zinc Transporter of Arabidopsis (van der Zaal *et al.*, 1999), was renamed *AtMTP1*. Overexpression in Arabidopsis enhances Zn resistance (van der Zaal *et al.*, 1999) while T-DNA insertion (Kobae *et al.*, 2004) or RNA interference-mediated silencing (Desbrosses-Fonrouge *et al.,* 2005) increases Zn sensitivity. *AtMTP3* is also thought to be involved in Zn transport (Arrivault *et al.,* 2006); *AtMTP11*, however, transports Mn (Peiter *et al.*, 2007; Delhaize *et al.*, 2007).

The rice ortholog *OsMTP1* was recently characterized by Yuan et al (2012) and Lan et al (2012). Located on chromosome 5, it is most highly expressed in mature leaves and stem (Yuan *et al.,* 2012). Both overexpression and RNAi-mediated silencing suggest a role for the transporter in zinc, cadmium and nickel movement, a hypothesis strengthened by functional complementation of yeast mutants (Yuan *et al.*, 2012). However, there is controversy over OsMTP1 localization, reported at the plasma membrane when expressed in onion epidermal cells (Yuan *et al.,* 2012) or at the vacuole when expressed in *Saccharomyces cerevisiae* (Lan et al., 2012).

Our study aims to clarify the membrane localization of OsMTP1 and investigate the importance of key residues in transport ability and specificity. We show here that OsMTP1 is localized to the vacuole when stably expressed *in planta*. We further demonstrate that OsMTP1 is a Zn transporter that can also transport Co, Fe and Cd. Moreover, we show that single-residue substitutions can alter substrate specificity.

Materials & Methods

Plant Material and Growth Conditions

Growth of rice plants (Nippombare cultivar) for leaf RNA extraction and *OsMTP1* amplification

Rice seeds of the Nipponbare cultivar were germinated for four days in an incubator at 28°C, on filter paper soaked with distilled water, and transferred to holders positioned over plastic pots with five liters of nutrient solution (16 seedlings per pot) containing 700 **µ**M K_2SO_4, 100 **µ**M KCl, 100 **µ**M KH_2PO_4, 2 mM $Ca(NO_3)_2$, 500 **µ**M $MgSO_4$, 10 **µ**M H_3BO_3, 0.5 **µ**M $MnSO_4$, 0.5 **µ**M $ZnSO_4$, 0.2 **µ**M $CuSO_4$, 0.01 **µ**M $(NH_4)_6 Mo_7O_{24}$ and 100 **µ**M Fe^{+3}-EDTA (as described by Kobayashi et al., 2005) . The pH of the nutrient solution was adjusted to 5.4 by addition of 0.5 mol/L NaOH. Plants were kept at 28°C ± 1°C under a photoperiod of 16h/8h light/dark

(150 **µ**mol m^{-2} s^{-1}) for 10 days. Solutions were replaced every 3 to 4 days and leaf samples were harvested for RNA extraction.

Growth of *Arabidopsis* plants (wt, *mtp1-1* mutant and *OsMTP1*-transformants)

The plants were grown in a controlled-environment growth room with a day night cycle (23°C 16 h light, 120 µmol m^{-2} s^{-1}; 18°C 8 h dark). Substrate composition contained vermiculite (medium size), seed and modular compost (F2+S) (Levington) at 1:1:1 (v/v) ratio, with 0.28 g/L Intercept insecticide (Bayer, Canada); sterilized by autoclaving at 121°C for 15 min at 1 bar pressure as previously described (Mills *et al.*, 2010). The T-DNA knockout *mtp1-1* mutant (Kobae *et al.*, 2004) was kindly provided by Professor Masayoshi Maeshima (Nagoya University, Japan).

Metal tolerance assay in Arabidopsis plants

These assays were similar to those previously described (Mills et al. 2008). *Arabidopsis thaliana* seed from wt (ecotype Wassilewskija), *mtp1-1* mutant and transgenic T3 homozygous lines overexpressing *OsMTP1* (lines 1 and 2) were sterilized in 15% (v/v) bleach for 15 min and rinsed five times with sterile water. They were then inoculated onto plates containing 0.8% (w/v) agarose (Melford), 1% (w/v) sucrose, and one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) with a range of Zn concentrations (provided as $ZnSO_4$), from 0 to 500 µM. Seed were stratified at 4°C for 48 h prior to transfer to a controlled-environment cabinet (22°C, 16 h light, 110 µmol m^{-2} s^{-1}; 18°C, 8 h dark) and plates were incubated vertically. Fresh weight and chlorophyll determinations were performed as described below.

Amplification and cloning of *OsMTP1*

The full-length sequence of *Oryza sativa OsMTP1* (accession no. AY266290/ LOC_Os05g03780) is found in GenBank™. Total RNA from rice leaves was extracted using the Concert Plant RNA Reagent (Invitrogen) and treated with DNaseI (Invitrogen). First-strand cDNA synthesis was performed with oligodT and reverse transcriptase (M-MLV, Invitrogen) using 1 µg of RNA. *OsMTP1* full-length coding sequence was amplified from cDNA with Pfu DNA Polymerase (Promega) using forward primer 5′CACCATGGACAGCCATAACTCAGCA and reverse primer 5′-CTACTCGCGCTCAATCTGAAT. In addition, reverse primer 5′-

CTCGCGCTCAATCTGAATG was used to amplify a fragment without the stop codon referred as non-stop (NS). PCR products were cloned into the entry vector pENTR/D-TOPO (Invitrogen), using gateway technology. Sequencing confirmed the nucleotide fidelity of *OsMTP1* in the entry vector

Expressing *OsMTP1* in the Arabidopsis *mtp1-1* mutant

Using gateway technology *OsMTP1*(NS) was transferred from the entry clone to the pMDC83 destination vector (for C-terminal GFP tagging), by LR recombination. Sequencing of expression clones was carried out to confirm correct transfer. Plasmids were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation and plants (T-DNA knockout *mtp1-1* mutant; Kobae *et al.*, 2004), were transformed using the floral dip method (Clough and Bent, 1998) but including a 3 h pre-induction of *vir* genes by addition of 100 μM acetosyringone to the culture before dipping. Homozygous T3 plants were used for analysis.

RT-PCR was used to confirm expression of *OsMTP1* in Arabidopsis. To do this, RNA was isolated from 11-day old seedlings grown on 0.5 MS agar plates. cDNA synthesis and RT-PCR were carried out as previously described (Mills *et al.*, 2008) except that the primers used to detect *OsMTP1* were forward primer 5′-CACCATGGACAGCCATAACTCAGCA and reverse primer 5′-CTACTCGCGCTCAATCTGAAT.

Fresh weight and chlorophyll measurements

Fresh weight and chlorophyll concentrations were determined for *Arabidopsis thaliana* seedlings of wt (ecotype Wassilewskija), *mtp1-1* mutant and T3 homozygous lines expressing *OsMTP1-GFP* (lines 1 and 2), as previously described by Mills et al. (2008). Seedlings were grown (as described above in the metal tolerance assays in Arabidopsis) on six separate plates for a range of Zn concentrations, each plate having 4 wild-type seedlings, 4 *mtp1-1* mutant seedlings and 4 of each transgenic line. Following growth, the seedlings were removed from the plates using forceps and weighed (four seedlings per genotype) and placed in an eppendorf for chlorophyll determination. Chlorophyll was determined after harvesting whole seedlings following extraction in *N*,*N*-dimethylformamide (DMF) (Moran and Porath, 1980). The data presented are from one experiment (conducted twice) and are the means from the six plates ± S.E. expressed on a per seedling basis.

***OsMTP1* constructs for yeast expression and site-directed mutagenesis**

The entry clones pENTR *OsMTP1* full length and pENTR *OsMTP1*(NS) (described above) were used to introduce the *OsMTP1* cDNAs into the yeast expression vector p426-EGFP (Alberti *et al.*, 2007) by LR recombination, gateway technology (Invitrogen). Site-directed mutagenesis using *OsMTP1*(NS) from the entry vector as the DNA template was performed using the QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primers are listed in supplemental data Table 1. All mutations were confirmed by DNA sequencing and recombined into p426-EGFP vector as described above. Constructs were transformed into *Saccharomyces cerevisiae*: wt BY4741 (*MATa, his3-1, leu2-0, met15-0, ura3-0*) and *zrc1 cot1* double mutant (*MATa; his3-1, leu2-0, met15-0, ura3-0, zrc1::natMX cot1::kanMX4)* for zinc and cobalt complementation analyses; wt DY150 (*MATa ura3-52, leu2-3, 112, trp1-1, his3-11, 15, ade2-1, can1-100(oc)*) and *ccc1* mutant (*MATa ura3-52, leu2-3, 112, trp1-1, his3-11, 15, ade2-1, can1-100(oc) Δccc1::HIS3*) for iron complementation analyses; and wt BY4741 and *ycf1* mutant (*MATa;his3-1; leu2-0; lys2-0;ura3D0; YDR135c::kanMX4*) for Cd complementation analyses. The Zn-hypersensitive *zrc1 cot1* mutant was obtained from Dr. U. Kramer (Ruhr University, Bochum).

Yeast transformation

Yeast transformation was performed using the LiOAc/PEG method (Gietz *et al.*, 1992). Transformants were selected on SC (Synthetic Complete) media without uracil (5 g L^{-1} ammonium sulphate, 1.7 g L^{-1} yeast nitrogen base, 1.92 g L^{-1} yeast synthetic drop-out media supplement without uracil; Sigma, UK) with 2% glucose (w/v) 2% (w/v) agar (Difco technical) (adjusted to pH 5.3 before addition of agar and prior to autoclaving). Plates were incubated at 30°C for 3 days.

Metal tolerance assays in yeast

For metal sensitivity tests, yeast cultures were grown overnight at 30°C in selective liquid medium, SC (Synthetic Complete) without uracil (described above). Overnight cultures were centrifuged for 4 minutes at 4000 rpm and resuspended in SC galactose liquid medium (pH 5.0) with 2% galactose (w/v) in place of glucose, further incubated for 4 hours (30°C, 200 rpm). Yeast cultures were diluted to the same OD600 (approx. 0.4) in SC liquid medium without uracil, 2% (w/v) galactose. Aliquots were inoculated onto SC without uracil, 2% (w/v) agar (Difco technical) 2% (w/v) galactose (adjusted to pH 5.3 before addition of agar and prior to autoclaving) containing various concentrations of different metals. Inoculated plates were

incubated at 30°C for 4-6 days.

Localization studies in yeast

Subcellular localization of wt and mutated proteins in yeast was assessed using in-frame C-terminal fusions with EGFP in the p426-EGFP vector. To induce expression of the fusion proteins, yeast cells were pre-grown overnight in SC media containing glucose to mid-log phase. The cells were switched to SC media containing galactose and grown for 24h, then were processed for fluorescence microscopy. The GFP fluorescence of yeast was observed using fluorescence microscopy under an Olympus FluoView 1000 confocal laser scanning system. EGFP emission was detected between 505-530 nm. Images were captured with a high-sensitivity photomultiplier tube detector.

Localization studies in *Arabidopsis thaliana*

Seeds of both T3 homozygous lines expressing *OsMTP1* with a C-terminal GFP tag (line 1 and 2) and wt (ecotype Wassilewskija), were surface sterilized in 15% (v/v) bleach for 15 min, rinsed five times with sterile water, and inoculated onto plates containing 0.8% (w/v) agarose (Melford), 1% (w/v) sucrose, and one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) as previously described (Mills *et al.*, 2008). Seed were stratified at 4°C for 48 h prior to transfer to a controlled-environment cabinet (22°C, 16 h light, 110 µmol m^{-2} s^{-1}; 18°C, 8 h dark) and plates were incubated vertically for seven days. Whole seedlings were placed on the slide in water and covered with cover slide for imaging using an Olympus FluoView 1000 confocal laser scanning system. GFP, propidium iodide (PI) and chlorophyll were excited using the 488 nm line of an argon ion laser. GFP and PI emission were detected between 505–530 nm and 610-650 nm, respectively; chlorophyll autofluorescence was detected using a LP580 filter. Cell wall staining was performed using propidium iodide (Invitrogen) using a 10 µg/ml solution in water. Plants were emerged in the solution for 5 minutes and rinsed three times with sterile water before analysis.

Bioinformatics analysis

OsMTP1 transmembrane domain arrangement was predicted using online helix-prediction programmes THMHMM (Krogh *et al.*, 2001) (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius (Kall *et al.*, 2004) (http://phobius.sbc.su.se/). The subsequent topology was created according to their predictions.

Sequence alignment and phylogenetic analyses for MTP1 proteins were performed using the

MEGA (Molecular Evolutionary Genetics Analysis) 4.1 package (Tamakura *et al.*, 2007). Protein multiple alignments were obtained with ClustalW and phylogenetic trees were reconstructed with the neighbor-joining method and the following parameters: pairwise deletion option, 1000 replicates of bootstrap and Poisson correction. The consensus tree shows only branches with a bootstrap consensus >50.

Results

Sequence analysis of OsMTP1

The full-length cDNA fragment of *OsMTP1* (accession no. AY266290 /LOC_Os05g03780) was amplified by high-fidelity RT-PCR based on GenBank™ and Rice Annotation Project information from NCBI (http://www.ncbi.nlm.nih.gov/nuccore). *OsMTP1* coding sequence consists of one exon, which encodes a protein with 418 amino acids. Percentage identities and similarities between OsMTP1, AtMTP1 and HvMTP1 are shown in Table 1. Highest identity (84%) and similarity (90%) scores at the amino acid level were observed between OsMTP1 and HvMTP1. We have generated a phylogenetic tree based on MTP1 amino acid sequences from six monocotyledons, *Sorghum bicolor*, *Zea mays*, *Setaria italica*, *Hordeum vulgare*, *Oryza sativa* and *Brachypodium distachyon*, plus *Arabidopsis thaliana* MTP1 and MTP3 sequences (Fig. 1). The phylogenetic tree shows that *Oryza sativa* has only one MTP1 sequence, while *Zea mays*, *Brachypodium distachyon* and *Arabidopsis thaliana* have two. Also, the appearance of new MTP1 sequences occurred after the speciation of either *Arabidopsis* or each monocot. OsMTP1 and HvMTP1 seem to be closely related. The multiple sequence alignment of AtMTP1, OsMTP1 and HvMTP1 proteins is shown in Figure 2; positions with a single, fully conserved residue are highlighted, as are those with conserved similar properties. Transmembrane domains (TMDs) predicted by Phobius (http://phobius.sbc.su.se/) are also marked in Figure 2. The proteins are predicted to possess cytoplasmic N- and C-termini and six TMDs, with a small cytoplasmic loop between TMDs II and III, and a large cytoplasmic, histidine-rich loop between TMDs IV and V; OsMTP1 possesses the loop with the most histidine residues, followed by HvMTP1. The 17 residues of CDF signature sequence (Montanini *et al.*, 2007) are identical for each sequence, also highlighted in Figure 2.

OsMTP1 transports Zn in *Arabidopsis thaliana*

The T-DNA insertion mutant of *A. thaliana* (strain Wassilewskija) *mtp1-1* is sensitive to elevated Zn, suffering stunted growth and pale coloration compared to wild-type ecotype Wassilewskija (Ws; Fig. 3A). These differences are significant upward of 100 µM Zn (Fig. 3B).

Resistance to Zn is restored when *mtp1-1* is transformed with *OsMTP1-GFP* under the control of the constitutive 35S promoter. Lines 1 and 2 are expressing a C-terminally GFP tagged version (35S:*OsMTP1-GFP*:*mtp1-1)*. GFP-tagging does not interfere with the rescue, indicating that the GFP-tagged construct can be used to determine the cellular localization of OsMTP1.

The effect on fresh weight and chlorophyll levels at varying Zn concentrations are shown in Figure 3B. Growth of all lines is impaired on 0 Zn compared to basal 15 μM Zn (Fig. 3B). The *mtp1-1* mutant shows reduced chlorophyll levels as Zn concentration is increased, compared to wt. Chlorophyll levels in transgenic lines follows the same trends as observed for fresh weight (Fig. 3B).

OsMTP1 site-directed mutations

The transmembrane data predicted by Phobius was used to construct the hypothetical membrane topology of OsMTP1 in Figure 4A. Six site -directed mutations were tested for their influence on the transport function of OsMTP1 (Figure 4B). We selected the residues based on conservation between CDF family members and also because mutation of some may confer a gain-of-function (Hoch *et al.*, 2012; Blaudez *et al.*, 2003; Lin *et al.*, 2009). Figure 4A also highlights the substitutions generated during site directed mutagenesis, to demonstrate their positions within the protein.

Zinc tolerance assay

Wild type and mutant *OsMTP1* cDNAs were cloned into the p426-EGFP vector, which fuses an EGFP-tag to the C-terminus of the gene to enable fluorescence localization studies (Alberti *et al.*, 2007). Here we used the *zrc1 cot1* yeast mutant, which shows a strong Zn susceptibility, for heterologous expression of *OsMTP1*.

OsMTP1 fully rescues the mutant phenotype at 10 mM Zn (Fig. 5). OsMTP1-EGFP also shows good rescue of the Zn-sensitive phenotype, with some growth on 10 mM Zn, but not quite as competently as the non-tagged OsMTP1. Compared to *OsMTP1-EGFP* construct, all of the mutations reduced the rescue of the Zn-sensitive phenotype to some extent, except R149G. This is obvious in L82S and L82F, but most apparent in H90D, which fails to rescue zinc-sensitivity of *zrc1 cot1* on 0.25 mM Zn (Fig. 5).

Cobalt tolerance assay

Yeast transformants *zrc1 cot1* were used in tests for Zn tolerance in parallel with Co tolerance. In contrast to the results from Yuan et al. (2012), we show that OsMTP1 does have Co transport function. OsMTP1 shows partial rescue of Co sensitivity at all concentrations tested (Fig. 6) but wild-type growth is far from being restored. The EGFP tag again impacts the rescuing ability of OsMTP1 (Fig. 6). The rescue of the Co-sensitive phenotype is less obvious than Zn. L82S again reduces the rescuing ability of OsMTP1whereas L82F, E145G, R149G and L317A improve Co transport relative to the non-mutated *OsMTP1-EGFP* construct.

Iron tolerance assay

The Fe transport-deficient *Saccharomyces cerevisiae ccc1* mutant lacks a vacuolar Fe sequestration function, demonstrating an Fe-sensitive phenotype at high Fe. Growth of *ccc1* transformed with an empty p426-EGFP vector is inhibited on 5 mM Fe compared to the wild-type DY150 (Fig. 7). OsMTP1 shows rescue of *ccc1* on 3.5 mM (data not shown) and 5 mM Fe (Fig. 7), indicating that OsMTP1 can transport iron in yeast. EGFP tagging slightly reduces the rescuing ability of OsMTP1 when compared to the non-tagged version of OsMTP1 (Fig. 7). L82F, H90D and R149G mutations improve Fe transport in *ccc1*. L82S slightly enhances the sensitive phenotype of ccc1, with poorer growth observed compared to OsMTP1-EGFP.

Cadmium tolerance assay

The Cd hypersensitive *Saccharomyces cerevisiae* mutant *ycf1* transformed with *OsMTP1* clearly confers tolerance to low levels of Cd (Fig 8), indicating that OsMTP1 can transport Cd in yeast. For all metals tested, expressing *OsMTP1-EGFP* consistently reduces transport when compared to the non-tagged construct; however, on Cd the reduction is more dramatic (Fig 8). All mutations were performed in the *OsMTP1-EGFP* construct and so their effects on Cd transport are not conclusive, but it seems that none of the mutations have an effect on Cd transport (Fig. 8). To confirm this possibility, L82F and H90D mutations were tested without EGFP tagging; no difference was noticed in *ycf1* complementation when compared to the non-mutated OsMTP1 (data not shown).

Manganese tolerance assay

OsMTP1 does not confer Mn tolerance to the Mn hypersensitive *Saccharomyces cervisiae* mutant *pmr1* (Fig. 9). Of all mutations tested we observed that L82F results in a gain of function for Mn complementation on low concentration (0.25mM Mn) (Fig. 9).

Summary of impact of site directed substitution on substrate specificity of OsMTP1

Table 2 summarizes the impact of site directed mutations on the functional complementation of metal-sensitive yeast mutants by OsMTP1. This includes the positioning of residues within the protein predicted by Phobius, also highlighted in Figure 2.

All mutations negatively impact Zn transport ability when compared to the non-mutated *OsMTP1-EGFP* construct, except for R149G; this mutation maintains Zn transport levels and enhances Co and Fe transport but has no effect on Cd and Mn complementation. L82S shows considerable reduction in Zn, Fe and Co transport ability. A similar reduction in growth was seen for G127S on Zn-containing media, although no further effect was seen on Co, Fe, Cd and Mn. All other substitutions have no effect on Cd, but enhance transport of Co or Fe: E145G and L317A both show increased survival on cobalt. Most notable mutations are L82F and H90D. L82F caused considerable reduction in Zn transport, failing to rescue *zrc1cot1* above 1mM Zn (Fig. 5) but resulting in a considerable increase of both Co and Fe transport and a gain of function for Mn. H90D appears to have abolished Zn transport with certainly no rescue of *zrc1cot1* on or above 0.25 mM Zn; the substitution shows enhanced survival on Fe.

OsMTP1 localises to the vacuole of *Saccharomyces cerevisiae*

In order to study the subcellular localization of OsMTP1, *zrc1 cot1* yeast cells expressing *OsMTP1-EGFP* construct were analyzed via confocal microscopy. Figure 10A illustrates the colocalization of *OsMTP1-EGFP* with vacuole organelles, which appear as a depression in the differential interference contrast (DIC) image. This result confirms that OsMTP1 localizes to the vacuole of *zrc1 cot1* cells, complementing the Zn hypersensitivity of the mutant (Fig. 5). The empty vector gives EGFP signal throughout the cytoplasm (Fig. 10B). Mutated versions of OsMTP1 with C-terminus EGFP tagging were also evaluated in *zrc1 cot1* cells to analyze whether the mutations could affect protein localization. These showed the same localization as the non-mutated OsMTP1 indicating that differences in transport function are not due to altered localization (Fig. S1).

OsMTP1 localises to the vacuolar membrane when stably expressed in *Arabidopsis thaliana.* Transgenic T3 lines of Arabidopsis *mtp1-1* mutant expressing *OsMTP1-GFP* were used to determine the membrane localisation of OsMTP1. The C-terminal tagged protein rescues *mtp1-1* Arabidopsis mutant similarly to the non-tagged OsMTP1 (data not shown), suggesting that GFP-tagging does not interfere with localisation. Confocal images show that

OsMTP1 is localised in the vacuolar membrane of cells (Fig. 11). Different cells were analyzed including cells in the root tip region containing small immature vacuoles (Fig. 11A), elongated root cells with a large central vacuole (Fig 11B-D), and cotyledon cells containing chloroplasts (Fig 11E). All show the same vacuolar localisation pattern.

Discussion

Micronutrient deficiency is a widespread problem in humans, exacerbated by the inherently low nutritional quality of cereal-rich staple diets and crop growth on suboptimal soils. Although required at relatively low concentrations in plant tissues, heavy metals such as Zn, Cu and Fe are equally important for the completion of a plant's lifecycle; crop yield is therefore severely impacted by growth on soils with low mineral phytoavailability. Additionally, along with other non-essential heavy metals such as Cd and lead (Pb), these micronutrients are toxic when present in excess, with plants exhibiting symptoms such as chlorosis, necrosis and growth inhibition (Marschner, 1995). For successful improvement of crop and subsequently human nutrition, we must understand the biological processes that govern uptake, homeostasis and distribution of each ion throughout the plant (Palmgren *et al.*, 2008; Mills *et al.,* 2010; Mills *et al.,* 2012; Mikkelsen *et al.,* 2012). Specialized transporters compartmentalize or efflux metal ions, maintaining cytosolic concentrations within a narrow range. CDFs/ MTPs are ubiquitous in all branches of life (Montanini et al. 2007; Gustin *et al.*, 2011). MTP1 genes have been cloned from a number of plant species and are shown to have a role in heavy metal transport (Kawachi *et al.*, 2008; Blaudez *et al.*, 2003; Kim *et al.*, 2004; Podar *et al.*, 2012). The Zn hyperaccumulator plant *Arabidopsis halerri* had a pentaplication of the *MTP1* gene during the evolutionary process, which is believed to have a role in Zn hypertolerance (Shahzad *et al.*, 2010). In this work, we investigate the transport function, localization and manipulation of the rice protein OsMTP1.

Transport function of OsMTP1

MTP1 transports zinc, cobalt, iron and cadmium in yeast

Expression of *OsMTP1* in *zrc1 cot1* fully rescues the Zn-sensitive phenotype, suggesting considerable Zn transporting ability of OsMTP1. This transporter also achieved partial rescue of *zrc1 cot1* on Co, *ccc1* on Fe and *ycf1* on Cd, confirming the ability to transport Co, Fe and Cd in yeast. Yuan et al (2012) failed to observe Co transporting ability of OsMTP1 when using the single mutant *cot1*. Results presented here suggest OsMTP1 is primarily a Zn transporter

but is also capable of transporting Co, Fe and Cd. OsMTP1 was also previously shown to transport Ni (Yuan *et al.*, 2012).

The OsMTP1 substrate specificity is considerably broader than that of the highly zinc-specific AtMTP1 (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005) and hybrid poplar PtdMTP1 (Blaudez *et al.*, 2003). CDF family members are classified into three major groups (Zn^{2+}, Fe^{2+}/Zn^{2+} and Mn^{2+}) based on their substrate specificity (Montanini *et al.*, 2007). OsMTP1 separates phylogenetically with the Zn^{2+} group, but members of this group do seem to be capable of transporting other metals. For example NtMTP1 (Shingu *et al.*, 2005) and HvMTP1 (Podar *et al.*, 2012) have increased cobalt affinity, while TcMTP1 from Zn/Cd-hyperaccumulating species *Thlaspi caerulescens* also transports non-essential Ni and Cd (Kim *et al.*, 2004). OsMTP1 is the only transporter reported to date that appears to have Fe transport activity: here we show it is able to reduce Fe hypersensitivity when expressed in the yeast strain *ccc1*. The Fe/Zn-CDFs group includes the bacterial FieF-like and the fungal MMT-like cluster. The MMT-like cluster comprises only fungal sequences with the exception of *S. cerevisiae* (Gaither and Eide, 2001). The plant family members of this group have not been studied to date to confirm this substrate specificity (Gustin *et al.*, 2011). The ability to rescue Fe hypersensitivity in yeast has not been evaluated for all plant MTP1 members characterized in the literature; PtdMTP1 (Blaudez *et al.*, 2003) and HvMTP1 (Podar *et al.*, 2012) were not tested. Shingu et al. (2005) suggested the broad substrate ability of NgMTP1 from hyperaccumulating *Nicotiana glauca* may be attributed to the number of histidine residues in the loop. OsMTP1 possesses more histidine residues in this loop than AtMTP1; perhaps this explains the wider substrate breadth of OsMTP1.

OsMTP1 **transports Zn in** ***Arabidopsis thaliana***

We also tested whether OsMTP1 could transport Zn when expressed in Arabidopsis. The Arabidopsis *AtMTP1* knockout line *mtp1-1* is sensitive to high Zn concentrations. Kawachi et al. (2009) evaluated the same line, demonstrating reduced germination rates and suppressed root and shoot growth on elevated Zn. The mutant phenotype is rescued, however, if transformed with *AtMTP1* under control of the constitutive CaMV 35S promoter (Kawachi *et al.*, 2009). Placed under the same promoter in the pMDC83 vector (Curtis and Grossniklaus, 2003), *OsMTP1* with a C-terminal GFP tag was also able to rescue the Zn-sensitive phenotype of *mtp1-1* (Fig. 3), suggesting conservation of function.

Our results confirm the findings of Kobae et al. (2004) and Kawachi et al. (2009): growth of the Arabidopsis T-DNA insertion mutant *mtp1-1* is inhibited on higher Zn concentrations, with

decreased fresh weight and chlorophyll content compared to the wild type. The *mtp1-1* Zn-sensitivity is displayed as stunted growth and poor photosynthetic ability. Kawachi et al. (2009) observed severe inhibition of growth in the mutant above 500 µM Zn and we show here that inhibition also occurs at lower levels (100 µM, 250 µM and 350 µM Zn) (Fig. 3).

Our results are consistent with OsMTP1 functioning as a Zn transporter *in planta*. 35S:*OsMTP1*-GFP:*mtp1-1* lines rescued the Zn-sensitive phenotype of the mutant with some lines achieving fresh weight and chlorophyll content comparable to that of the wt.

Lines that achieve best rescue of the sensitive phenotype show significantly lower fresh weight and chlorophyll values than the wt on zero Zn concentrations. Perhaps this higher level of expression results in the residual Zn being transported from the cytoplasm into the vacuole, causing a more deficient phenotype when extra Zn is not supplemented in the media. Yeast functional complementation assays suggest the substrate range of OsMTP1 also extends to other ions. Perhaps when Zn is in short supply, other metals are pumped into the vacuole, which may lead to deficient concentrations in the cytosol and subsequent detrimental effects.

OsMTP1 subcellular localisation

AtMTP1 (Kobae *et al.,* 2004), PtdMTP1 (Blaudez *et al.,* 2003) and HvMTP1 (Podar *et al.,* 2012) all target to the tonoplast when fused to a C-terminal GFP tag. However, Yuan et al. (2012) ascribed the localisation of OsMTP1 to the plasma membrane. In our study, OsMTP1 localisation in *zrc1 cot1* yeast mutant points to the vacuole (Fig. 10), a most reasonable result when comparing with MTP1 of other plant species. Further support for this comes from our data constitutively expressing *OsMTP1-GFP* in the Arabidopsis *mtp1-1* mutants (Fig. 11). The Zn hypersensitive phenotype of the Arabidopsis *mtp1-1* mutant was rescued by OsMTP1-GFP and confocal microscopy confirmed that OsMTP1-GFP is localised at the tonoplast. The localisation of OsMTP1 at the tonoplast is consistent with the vacuolar localisation observed for the closely related MTP1 from another cereal, barley (HvMTP1) (Podar *et al.*, 2012).

L82 and H90 play important roles in OsMTP1 substrate specificity

The yeast expression vector p426-EGFP has a C-terminal EGFP tag used to visualize OsMTP1 subcellular localization in yeast and evaluate the effect of site direct mutation in protein localization. Although reducing the transport function slightly, GFP-tagging did not change the specificity observed in the tests when compared with the non-tagged OsMTP1 protein. These results suggest that OsMTP1 is functionally active with the GFP protein tagged to the C-terminal region. In HvMTP1 (Podar *et al.*, 2012), PtdMTP1 (Blaudez *et al.,* 2003) and

AtHMA4 (Baekgaard *et al.*, 2010) the GFP tagging also slightly reduced the transport activity in yeast experiments but it did not change the response pattern. For all mutations of OsMTP1 studied here, the mutation did not affect targeting and OsMTP1 localised to the vacuole in all cases (Supplementary Fig S1).

Substitutions that entirely abolish the transporting function of a transporter tell us little about the importance of these residues in substrate specificity; loss of function may be independent of substrate change, such as destabilization of the tertiary structure (Lin *et al.*, 2009). Owing to their reduced or unchanged transporting abilities, G127S and L82S subsequently provide little information. L82F, however, maintaining the non-polar environment of leucine with phenylalanine, appears to be a gain-of-function mutant; the protein can still transport low levels of Zn, with an enhanced affinity for Fe, Co and Mn. L82 falls in the first non-cytoplasmic loop at the beginning of the CDF signature sequence (Montanini *et al.*, 2007). It is homologous to the L33F mutation that reduces Zn transport but increases Fe and Mn affinity in ScZRC1 (Lin *et al.*, 2009). It may, therefore, be concluded that L82 is an important residue for metal selectivity in plant CDFs as well as in yeast transporters.

Recent works (Podar *et al.*, 2012; Kawachi *et al.*, 2012), failed to discover a single substitution that entirely shifts the substrate specificity of AtMTP1 from Zn, suggesting instead that higher organisms regulate transport tightly by holding more than one residue responsible for the confinement to specific substrates (Podar *et al.*, 2012). However, we show here that H90D completely abolishes OsMTP1 Zn transport on the lowest concentration tested (0.3 mM Zn) while improving Fe transport. Key polar residues conserved in TMDs I, II, V and VI are thought to be involved in metal transport of CDF proteins (Gaither and Eide, 2001; Haney *et al.*, 2005), with putative Zn-binding sites within TMDII (Lin *et al.*, 2009; Cherezov *et al.*, 2008). H90, a polar histidine residue, falls within TMDII and the signature CDF sequence of OsMTP1. Homologous mutations in PtdMTP1 (H89K and H89A; Blaudez et al, 2003), in AtMTP1 (H90A; Kawachi et al, 2012) and in the mammalian metal transporters ZnT5 and ZnT8 (H451D and H106D, respectively; Hoch et al, 2012), also fall within the CDF signature sequence (Blaudez *et al.*, 2003; Montanini et al, 2007). These mutations abolish Zn transport, but the effect of the mutation on the transport of other metals besides Zn was not tested in PtdMTP1 or AtMTP1. These mutations in ZnT5 and ZnT8 allow Cd transport and so it was concluded that this motif was important in discriminating between Zn and Cd, and that metal selectivity is tuned by a coordination-based mechanism that raises the thermodynamic barrier to Cd (Hoch *et al.*, 2012). We show that this is not a general feature of CDFs/MTPs, as mutation of this residue in OsMTP1 does not lead to enhanced Cd transport, although it does

have a role in selectivity as it enhances Fe transport.

Three further mutations that cause less extreme substrate changes are E145G, R149G and L317A. E145G is homologous to E97G, a mutation in ScZRC1 that completely shifts the transported substrate from Zn to Fe and Mn (Lin *et al.*, 2009). Contrastingly, the corresponding AtMTP1 mutant just extends its transport ability to Co and Mn (Podar *et al.*, 2012) and when mutated to the non polar alanine, E145A, extends its transport to Co and Cd (Kawachi *et al.*, 2012). In none of the AtMTP1 studies cited above was the effect on Fe hypersensitivity investigated. In contrast to the results with ScZRC1, OsMTP1-E145G continues to transport Zn, at a slightly lower level than the non-mutated construct but it does show an increase in Fe transport. This mutation does markedly enhance cobalt transport. E145 is a polar residue falling within TMDIII of OsMTP1, a region containing conserved polar residues thought to be involved in metal transport across the family of CDF proteins (Gaither and Eide, 2001; Haney *et al.*, 2005). It may, therefore, be a further important residue for the specificity of OsMTP1.

OsMTP1 containing the R149G mutation does not show a significant difference in Zn tolerance from the wild type; enhancement of Co and Fe tolerance is evident, however, which may indicate a distinctive contribution of Arg149 to Zn selectivity over Co and Fe. The homologous mutation in ScZRC1 protein extends its transport to Fe and Mn (Lin *et al.*, 2009). In AtMTP1, mutation of the homologous residue to cysteine, another non-polar amino acid, conferred tolerance to high levels of Co and Cd (Kawachi *et al.*, 2012).

The OsMTP1 protein containing the L317A mutation also continues to transport Zn with slightly lower affinity, while enhancing Co transport. The homologous residue in PtdMTP1, L293, falls at the beginning of a Leu-zipper (LZ) motif, a repeating pattern of leucine residues at every seventh position that forms a functional alpha helix (Blaudez *et al.*, 2003). The major driving force of this motif is thought to be the bulky, hydrophobic side-chain of leucine residues (Luo *et al.*, 1999). Blaudez et al (2003) serially substituted leucine residues within the motif for alanine, maintaining the non-polar environment but losing the important side-chain. These mutations had an increasingly negative effect on the zinc-transport ability of PtdMTP1 further through the LZ motif; homologous mutation L293A had little impact but L314A, the final leucine in the motif, suffered a severe loss of Zn transport (Blaudez *et al.*, 2003). The leucine-zipper motif is conserved in a range of CDF proteins from a range of species; although not confirmed by Blaudez et al (2003), it also appears to be present in OsMTP1, running from L317 to L338 and falling across TMD-VI and the C-terminal tail. The last leucine of the leucine zipper motif is highly conserved in the CDF family. In AtMTP1, the L319 residue is

essential for protein function and is predicted to form dimerization contact between two protomers, in analogy to the corresponding residue L205 of EcYiiP (Kawachi *et al.*, 2012).

A recent work with AtMTP1 demonstrates that a mutation in L298, homologous to L317 in OsMTP1, also has little or no effect in Zn tolerance but conferred Co and Cd gain of function in yeast experiments (Kawachi *et al.*, 2012). These leucine residues seem to have an important role on ion selectivity that can be explained by AtMTP1 modelling experiments (Kawachi *et al.*, 2012). Although still small, the impact of L317A on the *zrc1 cot1* rescuing ability of OsMTP1 appears to be greater than that of the homologous residue in PtdMTP1; Co tolerance enhancement by the L317A mutation in the OsMTP1 protein is similar to AtMTP1 Co gain of function. It would be interesting to perform the same mutation on the successive leucines within the sequence. This study confirms the importance of L317 in the substrate specificity of OsMTP1, opening up opportunities to explore the importance of a hypothetical LZ motif.

MTP1 is expressed at considerably higher levels in hyperaccumulating species *A. halleri* and *T. caerulescens* than in related non-hyperaccumulating species (Shahzad *et al.*, 2010; Drager *et al.*, 2004; Gustin *et al.*, 2009b; Hanikenne and Nouet, 2011), enabling hypertolerance to zinc and cadmium. Residues that impart the metal sensitivity of OsMTP1, such as L82 and H90, may be important in altering the breadth of its transport for biofortification purposes.

To conclude, OsMTP1 is a vacuolar transporter that appears to transport Zn, but also Fe, Co and Cd perhaps with lower affinity. 35S:*OsMTP1*:*mtp1-1* fully rescues the zinc-sensitive phenotype of Arabidopsis knockout mutant *mtp1-1* and it would be interesting to also test Co and Fe transporting abilities *in planta*. H90 and L82 seem to be important residues for determining substrate specificity of OsMTP1. E145, R149 and L317 also appear to be related to substrate specificity. These new findings may be useful for defining strategies to generate plants suited for biofortification or phytoremediation applications.

Acknowledgements

We gratefully acknowledge Prof. Masayoshi Maeshima (Nagoya University, Japan) for *mtp1-1* mutant seeds and Prof. Ute Kramer (Ruhr University, Bochum) for the *zrc1cot1* mutant yeast strain. This research was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), research fellowship to JPF and scholarships to FKR and PKM; by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil), sandwich scholarship to PKM; and partly by funding to LEW from the European Union Framework Programme 6 as part of the Integrated Project Public Health impact of long-term, low level mixed element exposure in susceptible population strata (PHIME) (contract no

FOOD-CT-2006- 016253); it reflects only the authors' views. The Community is not liable for any use that may be made of the information contained therein. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Alberti S, Gitler AD, Lindquist S. 2007. A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. *Yeast* **24**, 913-919.

Arrivault S, Senger T, Kramer U. 2006. The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. *The Plant journal* **46**, 861-879.

Baekgaard L, Mikkelsen MD, Sorensen DM, Hegelund JN, Persson DP, Mills RF, Yang Z, Husted S, Andersen JP, Buch-Pedersen MJ, Schjoerring JK, Williams LE, Palmgren MG. 2010. A combined zinc/cadmium sensor and zinc/cadmium export regulator in a heavy metal pump. *The Journal of Biological Chemistry* **285**, 31243-31252.

Blaudez D, Kohler A, Martin F, Sanders D, Chalot M. 2003. Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. *The Plant cell* **15**, 2911-2928.

Cherezov V, Hofer N, Szebenyi DM, Kolaj O, Wall JG, Gillilan R, Srinivasan V, Jaroniec CP, Caffrey M. 2008. Insights into the mode of action of a putative zinc transporter CzrB in *Thermus thermophilus*. *Structure* **16**, 1378-1388.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735-743.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**, 462-469.

Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardson AE. 2007. A role for the AtMTP11 gene of Arabidopsis in manganese transport and tolerance. *The Plant Journal* **51**, 198-210.

Desbrosses-Fonrouge AG, Voigt K, Schroder A, Arrivault S, Thomine S, Kramer U. 2005. *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. *FEBS letters* **579**, 4165-4174.

Drager DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Kramer U. 2004. Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high MTP1 transcript levels. *The Plant Journal* **39**, 425-439.

Gaither LA, Eide DJ. 2001. Eukaryotic zinc transporters and their regulation. *Biometals* **14**, 251-270.

Gietz D, St Jean A, Woods RA, Schiestl RH. 1992. Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Research* **20**, 1425.

Gustin JL, Loureiro ME, Kim D, Na G, Tikhonova M, Salt DE. 2009. MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. *The Plant Journal* **57**, 1116-1127.

Gustin JL, Zanis MJ, Salt DE. 2011. Structure and evolution of the plant cation diffusion facilitator family of ion transporters. *BMC Evolutionary Biology* **11**, 76.

Hall JL, Williams LE. 2003. Transition metal transporters in plants. *Journal of Experimental Botany* **54**, 2601-2613.

Haney CJ, Grass G, Franke S, Rensing C. 2005. New developments in the understanding of the cation diffusion facilitator family. *The Journal of Industrial Microbiology and Biotechnology* **32**, 215-226.

Hanikenne M, Nouet C. 2011. Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. *Current Opinion in Plant Biology* **14**, 252-259.

Hoch E, Lin W, Chai J, Hershfinkel M, Fu D, Sekler I. 2012. Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn^{2+} over Cd^{2+} selectivity. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 7202-7207.

IRRI – International Rice Research Institute. Accessed in December 8th, 2012. Available at: http://www.irri.org/

Kall L, Krogh A, Sonnhammer EL. 2004. A combined transmembrane topology and signal peptide prediction method. *Journal of Molecular Biology* **338**, 1027-1036.

Kawachi M, Kobae Y, Mimura T, Maeshima M. 2008. Deletion of a histidine-rich loop of AtMTP1, a vacuolar Zn(2+)/H(+) antiporter of *Arabidopsis thaliana*, stimulates the transport activity. *The Journal of Biological Chemistry* **283**, 8374-8383.

Kawachi M, Kobae Y, Mori H, Tomioka R, Lee Y, Maeshima M. 2009. A mutant strain *Arabidopsis thaliana* that lacks vacuolar membrane zinc transporter MTP1 revealed the latent tolerance to excessive zinc. *Plant and Cell Physiology* **50**, 1156-1170.

Kawachi M, Kobae Y, Kogawa S, Mimura T, Kramer U, Maeshima M. 2012. Amino acid screening based on structural modeling identifies critical residues for function, ion selectivity and structure of Arabidopsis MTP1. *FEBS Journal* **279**, 2339-2356.

Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE. 2004. The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. *The Plant Journal* **39,** 237-251.

Kobae Y, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M. 2004. Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. *Plant and Cell Physiology* **45**, 1749-1758.

Kobayashi T, Suzuki M, Inoue H, Itai RN, Takahashi M, Nakanishi H, Mori S, Nishizawa NK.

2005. Expression of iron-acquisition-related genes in iron-deficient rice is coordinately induced by partially conserved iron-deficiency-responsive elements. *Journal of Experimental Botany* **56**, 1305–1316.

Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* **305**, 567-580.

Kurup S, Runions J, Köhler U, Laplaze L, Hodge S, Haseloff J. 2005. Marking cell lineages in living tissues. *The Plant Journal* **42**, 444–453.

Lan HX, Wang ZF, Wang QH, Wang MM, Bao YM, Huang J, Zhang HS. 2012. Characterization of a vacuolar zinc transporter OZT1 in rice (*Oryza sativa* L.). *Molecular Biology Reports* **40**, 1201-1210.

Lin H, Burton D, Li L, Warner DE, Phillips JD, Ward DM, Kaplan J. 2009. Gain-of-function mutations identify amino acids within transmembrane domains of the yeast vacuolar transporter Zrc1 that determine metal specificity. *Biochemical Journal* **422**, 273-283.

Luo Z, Matthews AM, Weiss SR. 1999. Amino acid substitutions within the leucine zipper domain of the murine coronavirus spike protein cause defects in oligomerization and the ability to induce cell-to-cell fusion. *Journal of Virology* **73**, 8152-8159.

Marschner H. 1995. Mineral nutrition of higher plants. San Diego: Academic Press. 889 p.

Mikkelsen MD, Pedas P, Schiller M, Vincze E, Mills RF, Borg S, Møller A, Schjoerring JK, Williams LE, Baekgaard L, Holm PB, Palmgren MG. 2012. Barley HvHMA1 is a heavy metal pump involved in mobilizing organellar Zn and Cu and plays a role in metal loading into grains. *Plos One* **7**, e49027.

Mills RF, Doherty ML, Lopez-Marques RL, Weimar T, Dupree P, Palmgren MG, Pittman JK, Williams LE. 2008. ECA3, a Golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in Arabidopsis. *Plant Physiology* **146**, 116-128.

Mills RF, Valdes B, Duke M, Peaston KA, Lahner B, Salt DE, Williams LE. 2010. Functional significance of AtHMA4 C-terminal domain in planta. *PLoS One* **5**, e13388.

Mills RF, Peaston KA, Runions J, Williams LE. 2012. HvHMA2, a P(1B)-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. *PLoS One* **7,** e42640.

Montanini B, Blaudez D, Jeandroz S, Sanders D, Chalot M. 2007. Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. *BMC Genomics* **8**, 107.

Moran R, Porath D. 1980. Chlorophyll determination in intact tissues using n,n-dimethylformamide. *Plant Physiology* **65**, 478-479.

Murashige T, Skoog FA. 1962. A revised medium for a rapid growth and bioassays with tobacco tissues cultures. *Plant Physiology* **15,** 473-479.

Nies DH, Silver S. 1995. Ion efflux systems involved in bacterial metal resistances. *Journal of Industrial Microbiology* **14**, 186-199.

Palmgren MG, Clemens S, Williams LE, Kramer U, Borg S, Schjorring JK, Sanders D. 2008. Zinc biofortification of cereals: problems and solutions. *Trends in Plant Science* **13**, 464-473.

Paulsen IT, Saier MH, Jr. 1997. A novel family of ubiquitous heavy metal ion transport proteins. *The Journal of Membrane Biology* **156**, 99-103.

Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJ, Blaudez D, Chalot M, Sanders D. 2007. A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 8532-8537.

Podar D, Scherer J, Noordally Z, Herzyk P, Nies D, Sanders D. 2012. Metal selectivity determinants in a family of transition metal transporters. *The Journal of Biological Chemistry* **287**, 3185-3196.

Shahzad Z, Gosti F, Frerot H, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P. 2010. The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. *PLoS Genetics* **6**, e1000911.

Shingu Y, Kudo T, Ohsato S, Kimura M, Ono Y, Yamaguchi I, Hamamoto H. 2005. Characterization of genes encoding metal tolerance proteins isolated from *Nicotiana glauca* and *Nicotiana tabacum*. *Biochemical and Biophysical Research Communications* **331**, 675-680.

Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**,1596-1599.

van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JA, Hooykaas PJ. 1999. Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. *Plant Physiology* **119**, 1047-1055.

Wirth J, Poletti S, Aeschlimann B, Yakandawala N, Drosse B, Osorio S, Tohge T, Fernie AR, Gunther D, Gruissem W, Sautter C. 2009. Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. *Plant Biotechnology Journal* **7**, 631-644.

Welch RM, Graham RD. 2004. Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of Experimental Botany* **55**, 353-364.

Yuan L, Yang S, Liu B, Zhang M, Wu K. 2012. Molecular characterization of a rice metal tolerance protein, OsMTP1. *Plant Cell Reports* **31**, 67-79.

Tables

Table 1. Analyses of protein sequence similarity at the amino acid level between AtMTP1, OsMTP1 and HvMTP1. Calculated using EMBOSS program Matcher. Scores are given as % similarity and % identity. Accession numbers: AtMTP1: NM130246; OsMTP1: AY266290; HvMTP1: AM286795.

Similarity\Identity	**AtMTP1**	**OsMTP1**	**HvMTP1**
OsMTP1	77.3	100	84.2
AtMTP1	100	69.4	67.7
HvMTP1	76.7	90.3	100

Table 2. Summarizing the effects of site directed mutations on OsMTP1 substrate selectivity. Amino acid substitutions within the OsMTP1 protein and their position relative to predicted transmembrane domains. Zinc and cobalt transporting abilities were assayed by complementation of *zrc1cot1* cells on $ZnSO_4$ and $CoCl_2$; iron transporting ability was assayed by complementation of *ccc1* cells on $FeSO_4$; cadmium transporting ability was assayed by complementation of *ycf1* cells on $CdCl_2$; and manganese transporting ability was assayed by complementation of *pmr1* cells on $MnCl_2$. X: reduction in growth compared to non-mutated OsMTP1; +: increase in growth; -: no difference in growth.

Mutation	**Location**	**Complementation of transport defect:**				
		Zn	**Co**	**Fe**	**Cd**	**Mn**
L82F	EL1	xx	++	+++	-	+
L82S	EL1	xxx	x	x	-	-
H90D	TMD2	xxxx	-	++	-	-
G127S	TMD3	x	-	-	-	-
E145G	TMD3	x	++	-	-	-
R149G	TMD3	-	+	+	-	-
L317A	TMD6	x	+	-	-	-

Figure legends

Figure 1: Phylogenetic tree showing the relationships between MTP1 protein sequences from six monocotyledons, *Brachypodium distachyon* (Bradi4g43740 and Bradi2g38330), *Hordeum vulgare* (HvMTP1), *Oryza sativa* (OsMTP1), *Setaria italica* (Si022161m.g), *Zea mays* (GRMZM2G036908 and GRMZM2G477741) and *Sorghum bicolor* (Sb09g002460), plus *Arabidopsis thaliana* MTP1 and MTP3 sequences. The tree was generated with MEGA 4.1 software. Bootstrap values from 1,000 replicates using the neighborjoining method are indicated at each node when the method agrees with the tree topology.

Figure 2: Multiple sequence alignment of *OsMTP1, HvMTP1* and *AtMTP1*. Generated by the ClustalW2 program. Black with white letters/ asterisk: fully conserved residues between sequences; dark grey with white letters/ colon: conservation of residues with strongly similar properties; pale grey with black letters/ full stop: conservation of residues with weakly similar properties. Transmembrane domains predicted by Phobius are marked by brown boxes. The CDF signature sequence is underlined in red.

Figure 3: Functional complementation of Arabidopsis knockout mutant *mtp1-1* by *OsMTP1* tagged to GFP**.** [A] representative 18 day old seedlings of each transformation, on low (15µM) and high (350µM) zinc-containing media. [B]: (i) bar chart of average fresh weight (mg) per seedling; significance calculated using paired T-test; #: significant difference to *mtp1-1*; *: significant difference to WS (wild-type *A. thaliana* ecotype Wassilewskija); black: WS; dark grey: *mtp1-1*; pale grey: 35S:*OsMTP1GFP*:*mtp1-1* line 1; white: 35S:*OsMTP1GFP*:*mtp1-1* line 2. (ii) bar chart of total chlorophyll content (µg) per seedling; significance calculated using paired T-test. See part (i) for explanation of keys.

Figure 4: A, Hypothetical membrane topology of OsMTP1 predicted according to the Phobius program. Residues highlighted in black are OsMTP1 sites chosen for directed mutations. EL, extracytosolic loop; IL, intracytosolic loop. B, substitutions resulting from site directed mutations in OsMTP1.

Figure 5: Functional analysis of *OsMTP1* in zinc-sensitive *Saccharomyces cerevisiae* mutant *zrc1cot1*. BY4741 was transformed with p426EGFP empty vector (e.v.); z*rc1 cot1* was transformed with p426EGFP vector either empty or expressing *OsMTP1* with or without stop codon (*). L82S to L317A refer to site directed substitutions generated within *OsMTP1*

without stop codon (*). Numbers represent serial dilutions of yeast cells in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with different concentrations of $ZnSO_4$. Plates were incubated at 28°C for 5 days.

Figure 6: Functional analysis of *OsMTP1* in cobalt-sensitive *Saccharomyces cerevisiae* mutant *zrc1 cot1*. BY4741 was transformed with p426EGFP empty vector (e.v.); z*rc1 cot1* was transformed with p426EGFP vector either empty or expressing *OsMTP1* with or without stop codon (*). L82S to L317A refer to site directed substitutions generated within *OsMTP1* without stop codon (*). Numbers represent serial dilutions of yeast cells in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with different concentrations of $CoCl_2$. Plates were incubated at 28°C for 5 days.

Figure 7: Functional complementation of iron-sensitive *Saccharomyces cerevisiae* mutant *ccc1*. DY150 was transformed with p426EGFP empty vector (e.v.); *ccc1* was transformed with p426EGFP vector either empty or containing *OsMTP1* with or without stop codon (*). L82S to L317A refer to site directed substitutions generated within *OsMTP1* without stop codon (*). Numbers represent serial dilutions of yeast cells in in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with 5mM of $FeSO_4$. Plates were incubated at 28°C for 5 days.

Figure 8: Functional complementation of cadmium-sensitive *Saccharomyces cerevisiae* mutant *ycf1*. BY4741 was transformed with p426EGFP empty vector (e.v.); *ycf1* was transformed with p426EGFP vector either empty or containing *OsMTP1* without stop codon (*). L82S to L317A refer to site directed substitutions generated within *OsMTP1* without stop codon (*). Numbers represent serial dilutions of yeast cells in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with different concentrations of $CdCl_2$. Plates were incubated at 28°C for 5 days.

Figure 9: Functional complementation of manganese-sensitive *Saccharomyces cerevisiae* mutant *pmr1*. K601 was transformed with p426EGFP empty vector (e.v.); *pmr1* was transformed with p426EGFP vector either empty or containing *OsMTP1* without stop codon (*). L82S to L317A refer to site directed substitutions generated within *OsMTP1* without stop

codon (*). Numbers represent serial dilutions of yeast cells in in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with 0.25 mM of $MnCl_2$. Plates were incubated at 28°C for 5 days.

Figure 10: (A) OsMTP1 localises to the vacuoles of *zrc1 cot1* yeast cells expressing *OsMTP1-EGFP*, as shown via confocal microscopy. The OsMTP1-EGFP (green) colocalises with vacuole organelles, which appear as a depression in the DIC image. (B) EGFP alone is distributed throughout the cytoplasm.

Figure 11: OsMTP1 vacuolar membrane localisation in *mtp1-1 Arabidopsis thaliana* mutant overexpressing *OsMTP1:GFP*. The red fluorescence is caused by cell walls stained with propidium iodide (A-D). GFP vacuolar fluorescence (green) in cells of the root tip region, containing small immature vacuoles (A); in elongated root cells with a large central vacuole (B-D); and in cotyledon cells containing chloroplasts (arrow) (E).

Figure 1:

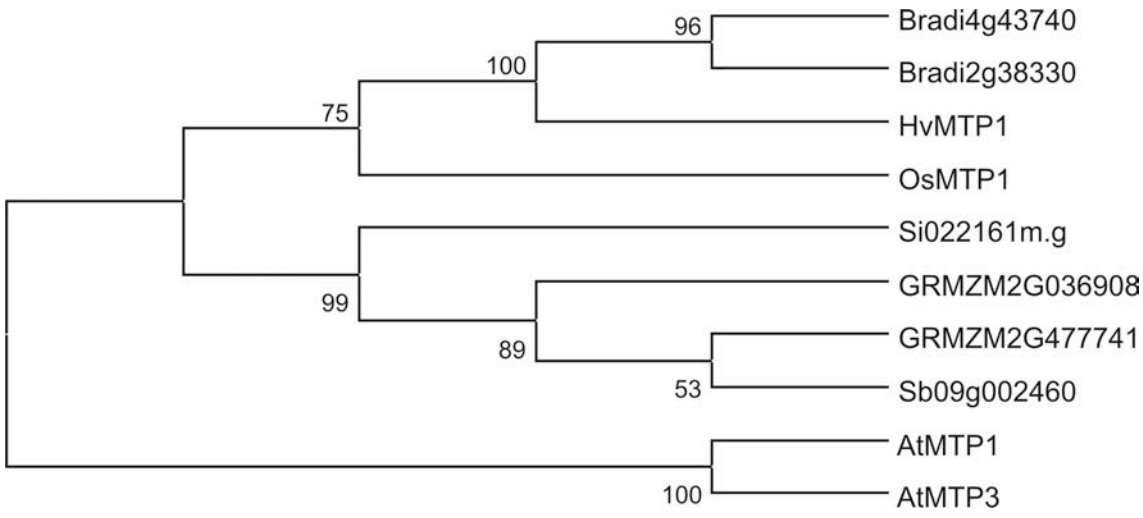

96
100
75
99
89
53
100
Bradi4g43740
Bradi2g38330
HvMTP1
OsMTP1
Si022161m.g
GRMZM2G036908
GRMZM2G477741
Sb09g002460
AtMTP1
AtMTP3

Figure 2:

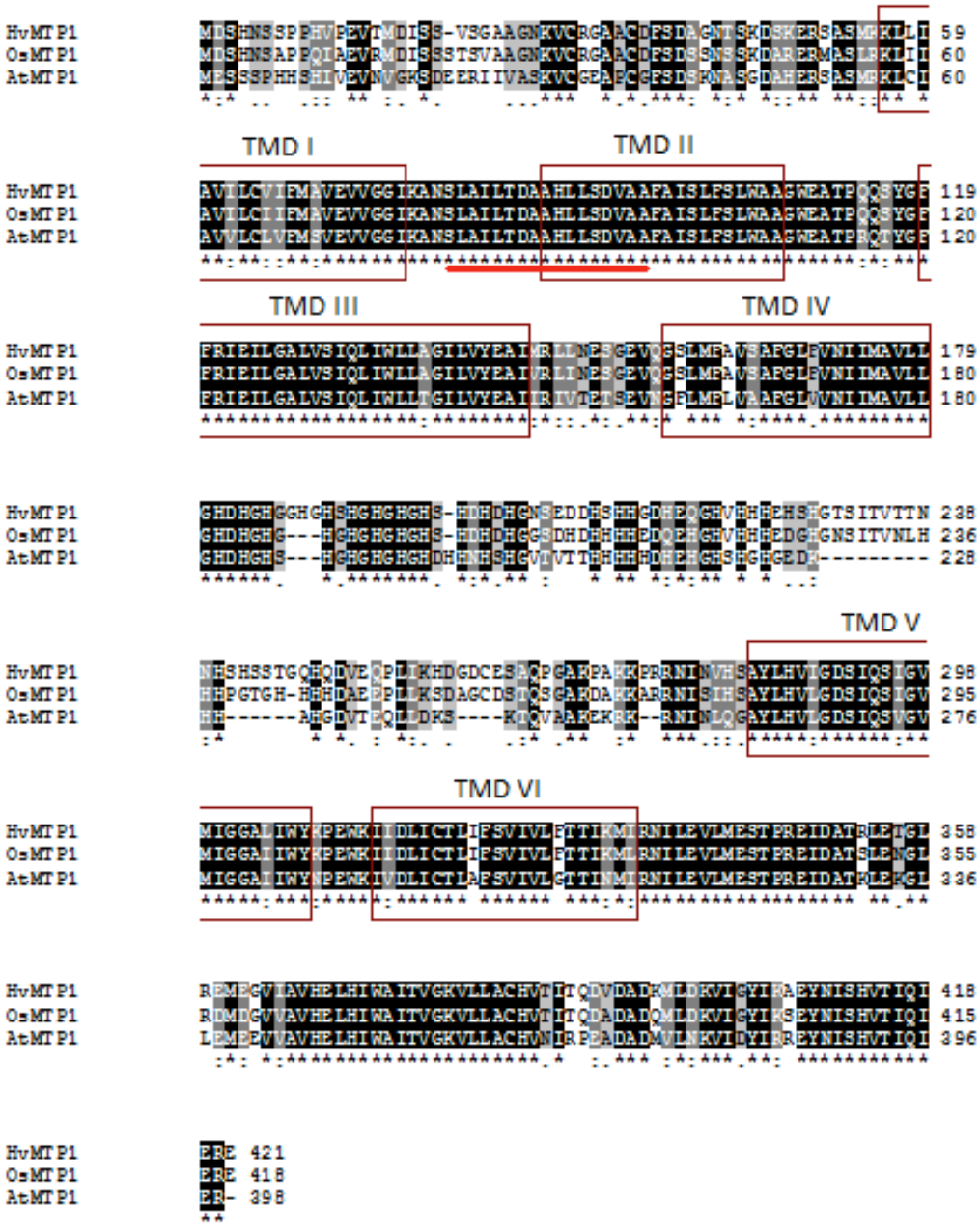
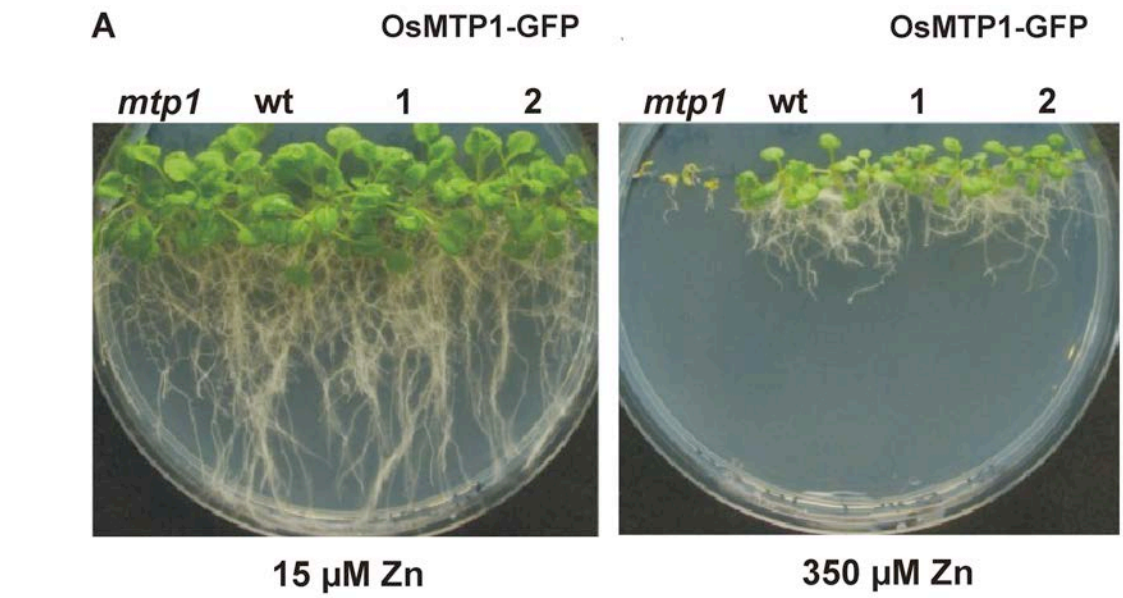


Figure 3:


A
OsMTP1-GFP
OsMTP1-GFP
mtp1 wt 1 2
mtp1 wt 1 2
15 µM Zn
350 µM Zn

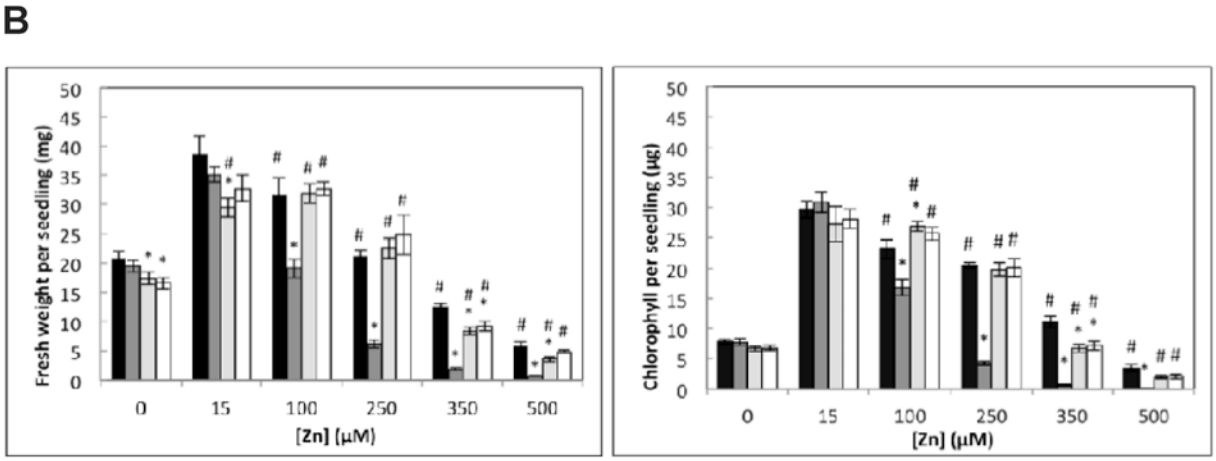
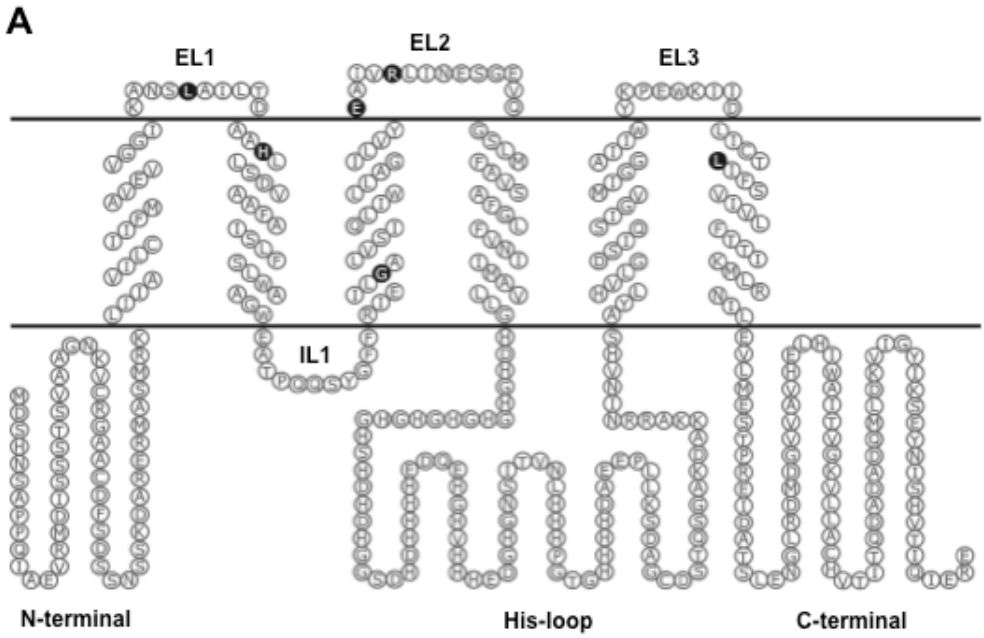

B
Fresh weight per seedling (mg)
Chlorophyll per seedling (µg)
[Zn] (µM)
0
15
100
250
350
500

Figure 4:



B

Position	EL1	TMD2	TMD3	EL2	TMD6
Substitution	L82F L82S	H90D	G127S	E145G R149G	L317A

Figure 5:

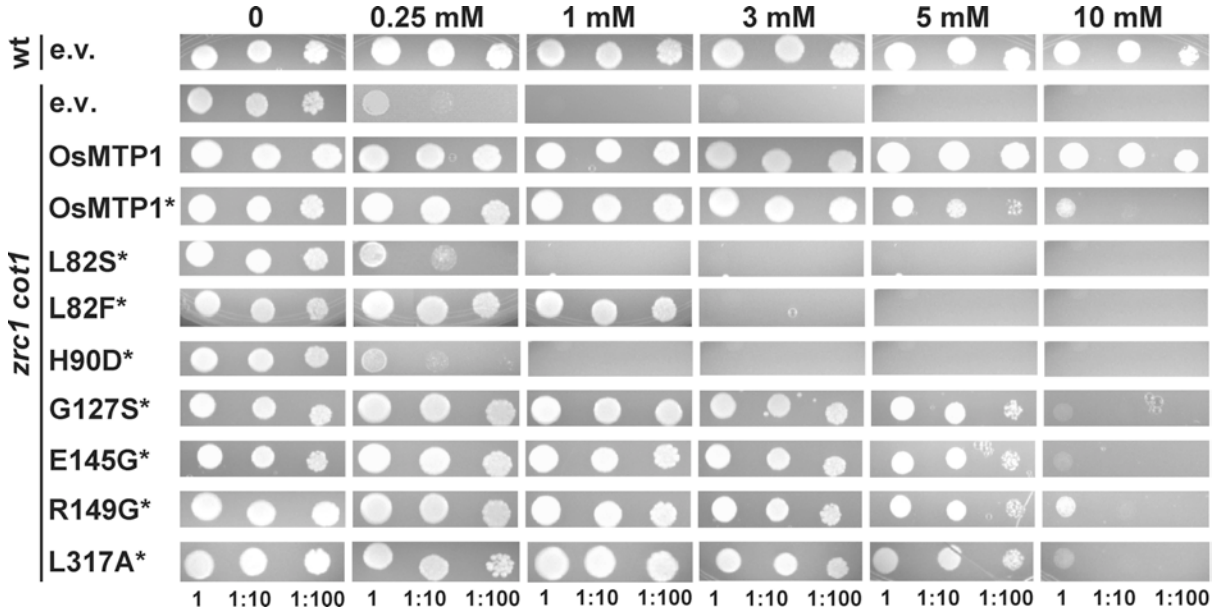

0
0.25 mM
1 mM
3 mM
5 mM
10 mM
wt
e.v.
zrc1 cot1
e.v.
OsMTP1
OsMTP1*
L82S*
L82F*
H90D*
G127S*
E145G*
R149G*
L317A*
1
1:10
1:100

Figure 6:

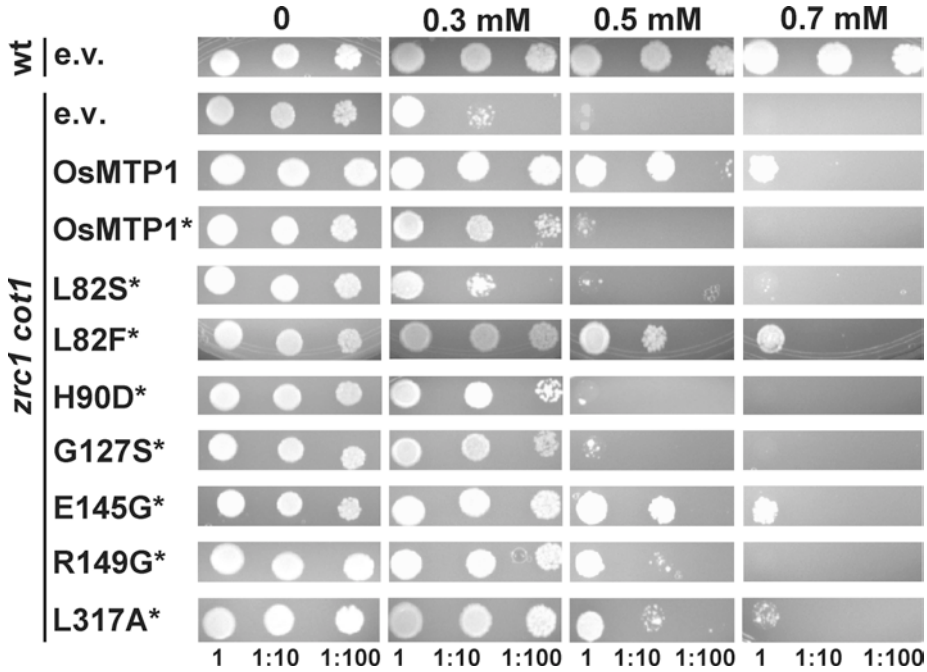

0
0.3 mM
0.5 mM
0.7 mM
wt
e.v.
zrc1 cot1
e.v.
OsMTP1
OsMTP1*
L82S*
L82F*
H90D*
G127S*
E145G*
R149G*
L317A*
1 1:10 1:100 1 1:10 1:100 1 1:10 1:100 1 1:10 1:100

Figure 7:

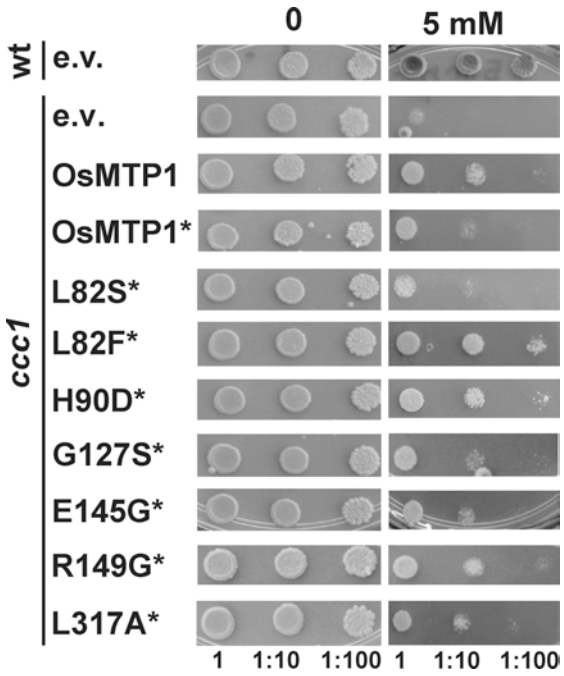

0
5 mM
wt
e.v.
ccc1
e.v.
OsMTP1
OsMTP1*
L82S*
L82F*
H90D*
G127S*
E145G*
R149G*
L317A*
1
1:10
1:100
1
1:10
1:100

Figure 8:

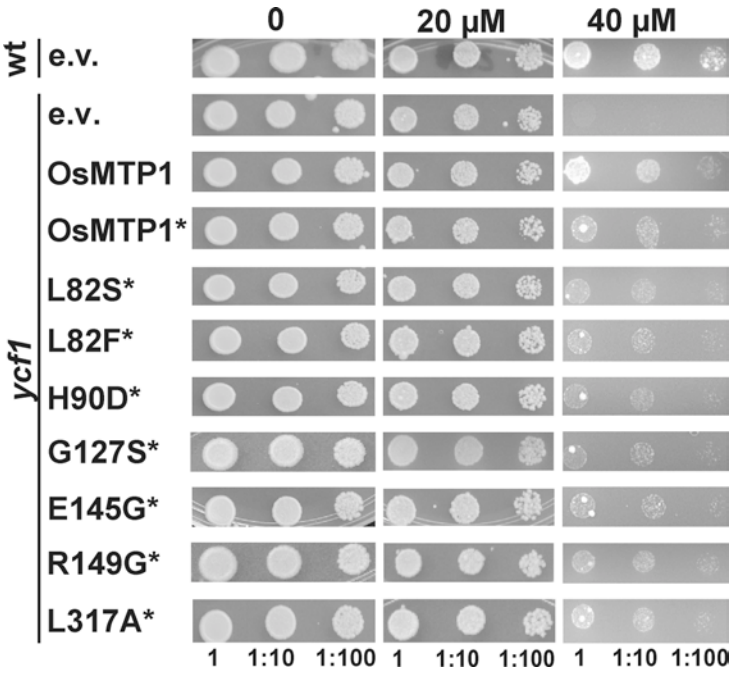

0
20 µM
40 µM
wt
e.v.
ycf1
e.v.
OsMTP1
OsMTP1*
L82S*
L82F*
H90D*
G127S*
E145G*
R149G*
L317A*
1 1:10 1:100 1 1:10 1:100 1 1:10 1:100

Figure 9:

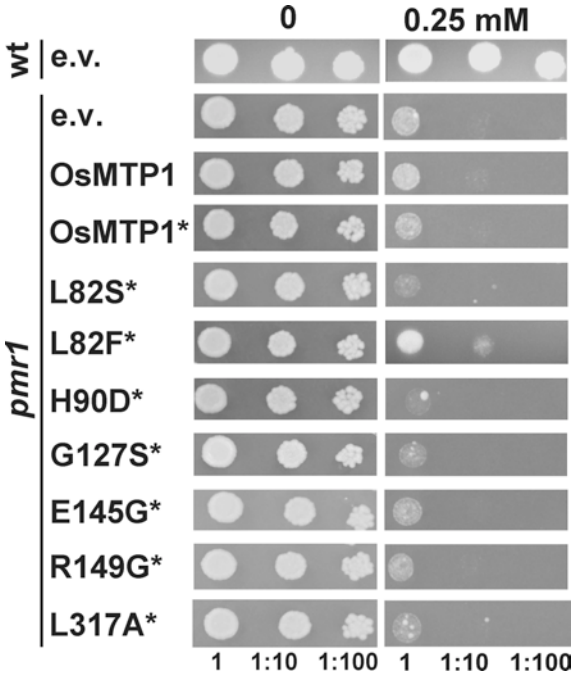

0
0.25 mM
wt
e.v.
pmr1
e.v.
OsMTP1
OsMTP1*
L82S*
L82F*
H90D*
G127S*
E145G*
R149G*
L317A*
1
1:10
1:100
1
1:10
1:100

Figure 10:

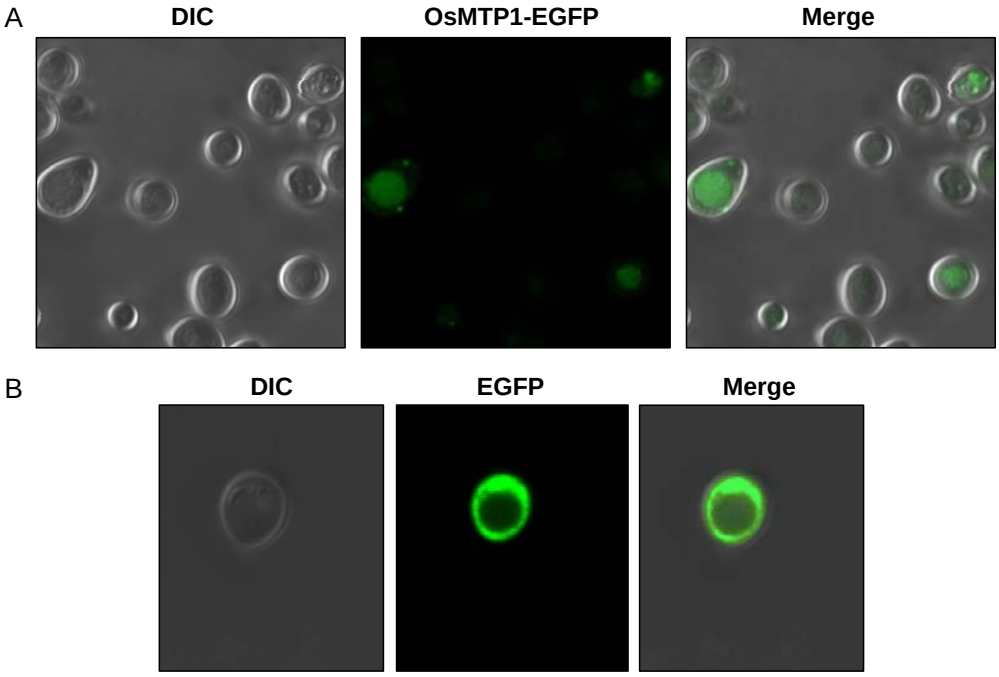
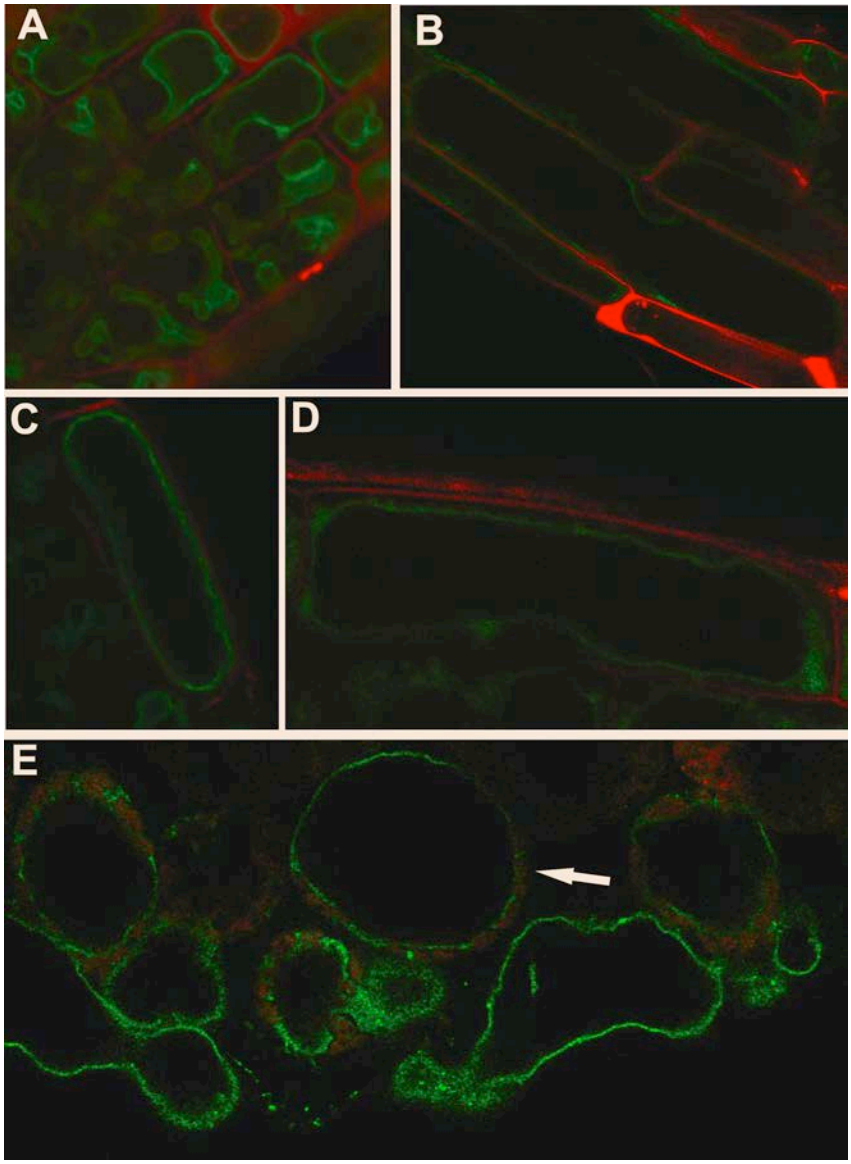


Figure 11:



Functional analysis of the rice vacuolar zinc transporter OsMTP1.

Paloma K. Menguer, Emily Farthing, Kerry A. Peaston, Felipe Klein Ricachenevsky, Janette Palma Fett, Lorraine E. Williams

Supplementary material

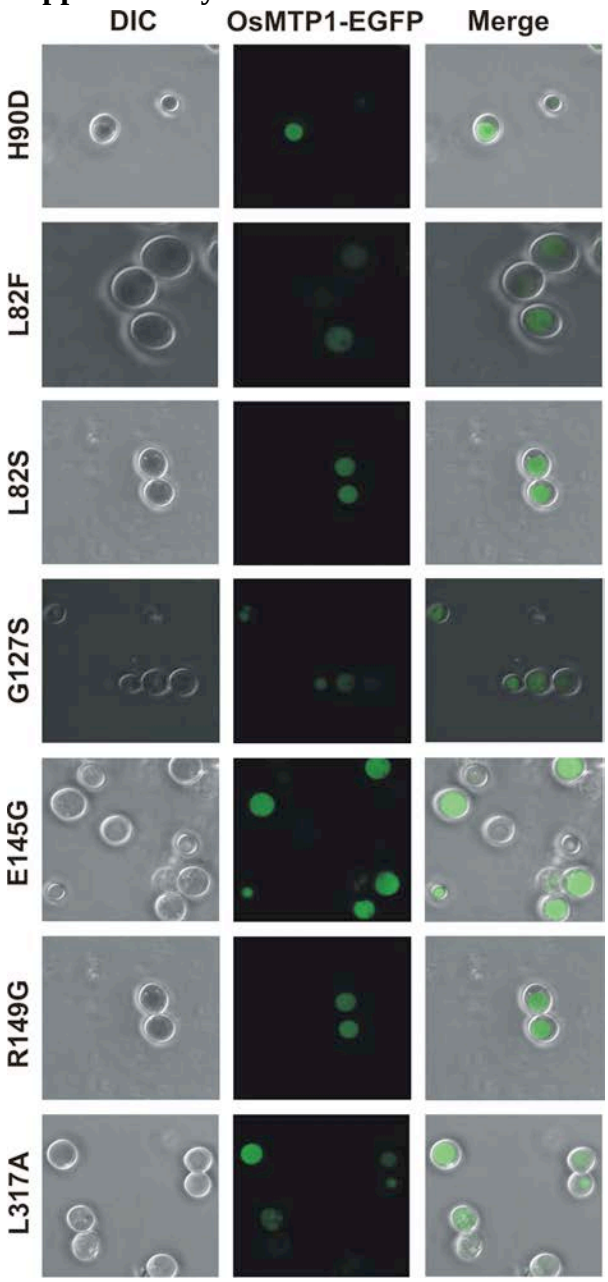


Figure S1: OsMTP1 site-directed mutations (H90D, L82F, L82S, G127S, E145G, R149 and L317A) with C-terminus EGFP tagging were analyzed for subcellular localization in *zrc1 cot1* yeast cells as shown via confocal microscopy. EGFP signal (green) from all mutated versions of OsMTP1 colocalizes with vacuole organelles, which appear as a depressions in the DIC images.

Table S1: List of primers used for cloning OsMTP1 site-directed mutations.

Name	Sequence
OsMTP1 H90D_F	CTTGACTGATGCAGCCGATCTCCTTTCGGATGT
OsMTP1 H90D_R	ACATCCGAAAGGAGATCGGCTGCATCAGTCAAG
OsMTP1 G127S_F	GGGTTTTTCCGTATAGAAATTCTTAGTGCCCTGGTTTC
OsMTP1 G127S_R	GAAACCAGGGCACTAAGAATTTCTATACGGAAAAACCC
OsMTP1 L82S_F	GGAGGTATCAAAGCAAACAGTTCGGCAATCTTGACTGATG
OsMTP1 L82S_R	CATCAGTCAAGATTGCCGAACTGTTTGCTTTGATACCTCC
OsMTP1 L82F_F	TTGGAGGTATCAAAGCAAACAGTTTCGCAATCTTGACTG
OsMTP1 L82F_R	CAGTCAAGATTGCGAAACTGTTTGCTTTGATACCTCCAA
OsMTP1 E145G_F	CTTGCTGGTATTCTTGTCTATGGAGCTATTGTAAGGCTCATTA
OsMTP1 E145G_R	TAATGAGCCTTACAATAGCTCCATAGACAAGAATACCAGCAAG
OsMTP1 L317A_F	TATTGATCTCATCTGCACCGCCATCTTCTCCGTGATCGTA
OsMTP1 L317A_R	TACGATCACGGAGAAGATGGCGGTGCAGATGAGATCAATA

Capítulo 2

Artigo a ser submetido ao periódico *Plos One*

(Fator de Impacto JCR 2011 = 4.092)

Molecular characterization of OsMTP11, a rice metal tolerance protein.

Paloma K. Menguer[1], Kerry A. Peaston[2], Felipe Klein Ricachenevsky[3], Raul Antônio Sperotto[4], Janette Palma Fett[1,3], Lorraine E. Williams[2]

[1]Departamento de Botânica, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, Porto Alegre, 91501-970, Brazil.

[2]Centre for Biological Science, University of Southampton, Life Sciences Building 85, Highfield Campus, Southampton SO17 1B, Hampshire, United Kingdom.

[3]Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, P.O.Box 15005, Porto Alegre, 91501-970, Brazil.

[4]Centro de Ciências Biológicas e da Saúde, Centro Universitário UNIVATES, Rua Avelino Tallini 171, Lageado, 95900-000, Brazil.

Paloma Koprovski Menguer paloma.menguer@gmail.com

Felipe Klein Ricachenevsky felipecruzalta@yahoo.com.br

Raul Antônio Sperotto raulsperotto@yahoo.com.br

Janette Palma Fett jpfett@cbiot.ufrgs.br

Lorraine E. Williams L.E.Williams@soton.ac.uk

Tel: +44(0) 23 80594278 Fax: +44(0) 23 8059 5159

Abstract

Heavy metal homeostasis is maintained in plant cells by specialized transporters, which compartmentalise or efflux metal ions, maintaining cytosolic concentrations within a narrow range. OsMTP11 is a member of the CDF/MTP family of metal cation transporters in *Oryza sativa*, which is closely related to *Arabidopsis thaliana* MTP11. When heterologously expressed in the yeast mutant *pmr1*, *OsMTP11* partially complemented its Mn-hypersensitivity. OsMTP11 alleviated, to some extent, the Zn sensitivity of *zrc1 cot1* mutant. These results suggest that OsMTP11 transports Mn but also Zn, possibly with lower affinity. Site-directed mutagenesis studies revealed four substitutions in OsMTP11, which appear to alter the transport activity of OsMTP11. OsMTP11 harbouring a substitution of leucine 150 to a serine fully rescued *pmr1* Mn-sensitivity in all concentrations tested. All the other substitutions reduced *pmr1* complementation in different levels. The mutated amino acid residues seem to be important in transport function and may be a starting point for refining transporter activity. These are the first findings on OsMTP11 characterization described in the literature.

Keywords: cation diffusion facilitator; metal tolerance protein; *Oryza sativa*; manganese transporter, site-directed mutations

Abbreviations: CDFs, Cation Diffusion Facilitators; DMF, *N*,*N*-dimethylformamide; MTPs, Metal Tolerance Proteins; TMDs, Transmembrane Domains.

Introduction

The proteins of the CDF (cation diffusion facilitator) family are involved in mechanisms of metal tolerance. According to phylogenetic analyzes, groups 8 and 9 of the CDF superfamily have an ancient origin, containing both prokaryote and eukaryote sequences. Products of a possible duplication event, these two distinct groups have been functionally characterized as Mn transporters (Gustin *et al.*, 2011). ShMTP8 from *Stylosanthes hamata* was the first member of this group to be studied. It is localized at the vacuolar membrane and is involved in the influx of Mn^{+2} into the vacuoles (Delhaize et al., 2003; Delhaize *et al.*, 2007).

AtMTP11, a member of the Mn-CDF group 9 in *Arabidopsis thaliana*, conferred enhanced Mn^{2+} tolerance and to a lesser extent copper (Cu^{2+}) tolerance when expressed in yeast cells, but no increased tolerance to a range of other metals, as Zn^{2+}, Co^{2+} or Ni^{2+}, has been detected. Using yeast microsomal membrane vesicles, it was shown that AtMTP11 also confers Mn^{2+}-dependent proton-transport activity (Delhaize *et al.*, 2007). Differently from ShMTP8, AtMTP11 was not located at the vacuolar membrane, but was found in pre-vacuolar compartments or trans-Golgi (Delhaize *et al.*, 2007; Peiter *et al.*, 2007). In this way, the Mn tolerance conferred by AtMTP11 probably relies on vesicular trafficking and exocytosis of excess Mn^{2+} (Peiter *et al.*, 2007), which is corroborated by the increased accumulation of Mn in leaves of *Arabidopsis* mutant plants with disrupted *AtMTP11* gene (*mtp11*). The same *Arabidopsis* mutant plants are hypersensitive to elevated levels of Mn, whereas over-expression plants are hypertolerant (Delhaize *et al.* 2007; Peiter *et al.*, 2007).

Interestingly, two homologous genes from poplar, *PtMTP11.1* and *PtMTP11.2*, are also targeted to a Golgi-like compartment and are able to complement *mtp11 Arabidopsis* mutant plants, suggesting that poplar and *Arabidopsis* MTP11 proteins play similar functions (Peiter *et al.* 2007). As previously stated by Gustin *et al.* (2011), it is clear that at least some of the CDF members of the groups 8 and 9 are important to Mn homeostasis, and the early bifurcation and further expansion of these groups represent the adaptive strategies that each plant species have developed to maintain Mn homeostasis and to deal with Mn toxicity.

There are no reports of rice genes homologous to the Arabidopsis *AtMTP11*. The aim of this study was to identify rice *MTP11* genes and to characterize OsMTP11 in two heterologous systems: *Arabidopsis thaliana*, analyzing the capacity of OsMTP11 to complement the *atmtp11* mutant; and *Saccharomyces cerevisiae*, testing the ability of OsMTP11 to complement yeast mutants defective in metal transport, also analyzing the importance of key residues in transport ability and specificity. OsMTP11 appear to transport Mn and perhaps Zn with lower affinity. We also show that key residues are crucial for metal responses conferred by OsMTP11 in yeast.

Material and Methods

Growth of rice plants (Nippombare cultivar) for leaf RNA extraction and *OsMTP11* amplification

Rice seeds of the Nipponbare cultivar were germinated for four days in an incubator at 28°C, on filter paper soaked with distilled water, and transferred to holders positioned over plastic pots with five liters of nutrient solution (16 seedlings per pot) containing 700 **µ**M K_2SO_4, 100 **µ**M KCl, 100 **µ**M KH_2PO_4, 2 mM $Ca(NO_3)_2$, 500 **µ**M $MgSO_4$, 10 **µ**M H_3BO_3, 0.5 **µ**M $MnSO_4$, 0.5 **µ**M $ZnSO_4$, 0.2 **µ**M $CuSO_4$, 0.01 **µ**M $(NH_4)_6 Mo_7O_{24}$ and 100 **µ**M Fe^{+3}-EDTA (as described by Kobayashi et al., 2005) . The pH of the nutrient solution was adjusted to 5.4 by addition of 0.5 mol/L NaOH. Plants were kept at 28°C ± 1°C under a photoperiod of 16h/8h light/dark (150 **µ**mol m^{-2} s^{-1}) for 10 days. Solutions were replaced every 3 to 4 days and leaf samples were harvested for RNA extraction.

Growth of *Arabidopsis* plants (wt, *mtp11* mutant and *OsMTP11*-transformants)

The plants were grown in a controlled-environment growth room with a day night cycle (23°C 16 h light, 120 µmol m^{-2} s^{-1}; 18°C 8 h dark). Substrate composition contained vermiculite (medium size), seed and modular compost (F2+S) (Levington) at 1:1:1 (v/v) ratio, with 0.28 g/L Intercept insecticide (Bayer, Canada); sterilized by autoclaving at 121°C for 15 min at 1 bar pressure as previously described (Mills *et al.*, 2010).

Metal tolerance assay in Arabidopsis plants

Arabidopsis thaliana seeds from wt (ecotype Wassilewskija), *mtp11* mutant and transgenic T2 heterozygous lines overexpressing *OsMTP11* were sterilized in 15% (v/v) bleach for 15 min, rinsed five times with sterile water, and inoculated onto plates containing 0.8% (w/v) agarose (Melford), 1% (w/v) sucrose, and one-half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) as previously described (Mills *et al.*, 2008). The final metal concentration tested was 50 µM (control) and 1 mM (high) of $MnCl_2$. Seed were stratified at 4°C for 48 h prior to transfer to a controlled-environment cabinet (22°C, 16 h light, 110 µmol m^{-2} s^{-1}; 18°C, 8 h dark) and plates were incubated vertically.

Amplification and cloning of *OsMTP11*

The full-length sequence of *Oryza sativa OsMTP11* (LOC_Os01g62070) was found in the data bases from the Rice Genome Annotation Project. Total RNA from rice leaves was extracted

using the Concert Plant RNA Reagent (Invitrogen) and treated with DNaseI (Invitrogen). First-strand cDNA synthesis was performed with oligo dT and reverse transcriptase (M-MLV, Invitrogen) using 1 µg of RNA. *OsMTP11* full-length coding sequence was amplified from cDNA with Pfu DNA Polymerase (Promega) using forward primer 5′ CACCCCCTGGTTCGTGGAATAATG and reverse primer 5′- CTATTTTTCATGGGACAGAGCG. In addition, reverse primer 5′- TTTTTCATGGGACAGAGCGT was used to amplify a fragment without the stop codon referred as non-stop (NS). PCR products were cloned into the entry vector pENTR/D-TOPO (Invitrogen), using gateway technology. Sequencing confirmed the nucleotide fidelity of *OsMTP11* in the entry vector

Expressing *OsMTP11* in Arabidopsis *mtp11* mutant plants

Using gateway technology, *OsMTP11* full length and *OsMTP11*(NS) were transferred from entry clones to destination vectors pMDC32 and pMDC83 (C-terminal GFP tagging) respectively, by LR recombination. Sequencing of expression clones was carried out to confirm correct transfer. Plasmids were transformed into *Agrobacterium tumefaciens* GV3101 by electroporation and plants (T-DNA knockout *atmtp11* mutant), were transformed using the floral dip method (Clough and Bent, 1998) but including a 3 h pre-induction of *vir* genes by addition of 100 µM acetosyringone to the culture before dipping. Heterozygous T2 plants were analysed.

Fresh weight and chlorophyll measurements of seedlings <u>(future experiment)</u>

Fresh weight and chlorophyll concentrations were determined as previously described (Mills *et al.*, 2008). For each experiment, seedlings were grown on six separate plates per concentration, each plate having 4 wild-type seedlings, 4 *mtp11* mutant seedlings and 4 of each transgenic line. The data presented are the means from the six plates ± S.E. expressed on a per seedling basis. Chlorophyll was determined following extraction in *N,N*-dimethylformamide (DMF) (Moran and Porath, 1980).

***OsMTP11* constructs for yeast expression and site-directed mutagenesis**

The entry clone pENTR *OsMTP11* full length was used to introduce the *OsMTP11* cDNA into the yeast expression vector pYTV (Gon *et al.*, 2004) by LR recombination, gateway technology (Invitrogen). Site-directed mutagenesis using *OsMTP11* full length from the entry vector as the DNA template was performed using the QuikChange® II XL Site-Directed

Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Primers are listed in supplemental data Table S1. All mutations were confirmed by DNA sequencing and recombined into pYTV vector as described above. Constructs were transformed into *Saccharomyces cerevisiae*: wt BY4741 (*MATa, his3-1, leu2-0, met15-0, ura3-0*) and *zrc1 cot1* double mutant (*MATa; his3-1, leu2-0, met15-0, ura3-0, zrc1::natMX cot1::kanMX4)* for zinc and cobalt complementation analyses; and wt K601 (MAT a; his3-1; leu2-0; met15-0; ura3-0) and *1111pmr1* mutant (*MAT a; his3-1; leu2-0; met15-0; ura3-0; pmr1::kanMX4*) for Mn complementation analyses. The Zn-hypersensitive *zrc1 cot1* mutant was obtained from Dr. U. Kramer (Ruhr University, Bochum, Germany).

Yeast transformation

Yeast transformation was performed using the LiOAc/PEG method (Gietz *et al.*, 1992). Transformants were selected on SC (Synthetic Complete) media without uracil (5 g L^{-1} ammonium sulphate, 1.7 g L^{-1} yeast nitrogen base, 1.92 g L^{-1} yeast synthetic drop-out media supplement without uracil; Sigma, UK) with 2% glucose (w/v) 2% (w/v) agar (Difco technical) (adjusted to pH 5.3 before addition of agar and prior to autoclaving). Plates were incubated at 30°C for 3 days.

Metal tolerance assays in yeast

For metal sensitivity tests, yeast cultures were grown overnight at 30°C in selective liquid medium, SC (Synthetic Complete) without uracil (described above). Overnight cultures were centrifuged for 4 minutes at 4000 rpm and resuspended in SC galactose liquid medium (pH 5.0) with 2% galactose (w/v) in place of glucose, further incubated for 4 hours (30°C, 200 rpm). Yeast cultures were diluted to the same OD600 (approx. 0.4) in SC liquid medium without uracil, 2% (w/v) galactose. Aliquots were inoculated onto SC without uracil, 2% (w/v) agar (Difco technical) 2% (w/v) galactose (adjusted to pH 5.3 before addition of agar and prior to autoclaving) containing various concentrations of different metals. Inoculated plates were incubated at 30°C for 4-6 days.

Localization studies *in planta* (future experiment)

Seedlings of both 35S::*OsMTP11-GFP* expressing plants and wt Col-0 were grown as described in Kurup et al (2005). Plants were mounted on microscope slides in water for imaging using an Olympus FluoView 1000 confocal laser scanning system. GFP, propidium iodide (PI) and chlorophyll were excited using the 488 nm line of an argon ion laser. GFP and

PI emission were detected between 505–530 nm and 610-650 nm, respectively; chlorophyll autofluorescence was detected using a LP580 filter.

Cell wall staining was performed using propidium iodide (Invitrogen) imbibition in a 10 µg/ml solution in water. Plants were immersed in the solution for 5 minutes before analysis.

Bioinformatics analysis

OsMTP11 and OsMTP11.1 transmembrane domain arrangement was predicted using online helix-prediction programmes THMHMM (Krogh *et al.*, 2001) (http://www.cbs.dtu.dk/services/TMHMM/) and Phobius (Kall *et al.*, 2004) (http://phobius.sbc.su.se/). The subsequent topology was created according to their predictions.

Gene expression based on microarray meta-analysis

Only specific Affymetrix probes (Table S2) for rice *OsMTP11* and *OsMTP11.1* genes were used to analyze expression data from Rice Oligonucleotide Array Database (http://www.ricearray.org/). Only high quality arrays were used.

Results

Sequence and expression analysis of OsMTP11

The study of MTPs in rice is important to elucidate mechanisms involved in metal homeostasis in crops. Analyzing the rice genome, two sequences with high homology to *AtMTP11* were found, named *OsMTP11* and *OsMTP11.1*, with 78% and 67% of amino acid identity to *AtMTP11*, respectively. OsMTP11 protein was predicted to possess cytoplasmic N- and C-termini and six TMDs, with two small cytoplasmic loops between TMDs II - III and TMDs IV - V, respectively (Fig. 1A). OsMTP11.1 protein is predicted to possess cytoplasmic N- and C-termini and four TMDs, with one cytoplasmic loop between TMDs II – III (Fig. 1B).

The full-length cDNA fragment of *OsMTP11* (LOC_Os01g62070) was amplified by high-fidelity RT-PCR based on information from the Rice Annotation Project.The *OsMTP11* DNA consists of six exons, and encodes a protein with 415 amino acids. The attempts to amplify *OsMTP11.1* (LOC_ Os05g38670) full length cDNA, also based on information from the Rice Annotation Project, nevertheless didn't generate a fragment, even testing RNA templates obtained from different organs (shoot, panicle and root) in different conditions (Mn and Zn excess). The *OsMTP11.1* DNA consists of eight exons, and is predicted to encode a protein with 418 amino acids.

To analyze the expression pattern of *OsMTP11* and *OsMTP11.1* genes based on microarray

meta-analysis, we used the Rice Oligonucleotide Array Database. Affymetrix unique probes used for expression analyses of *OsMTP11* and *OsMTP11.1* are listed in supplementary data (Table S2). The available data on expression of genes in different organs of rice plants is shown in Figure 2. According to microarray data, *OsMTP11* was highly expressed in shoots, particularly in leaves, and less expressed in seeds and reproductive tissues (Fig. 2). In agreement with our attempt to amplify its coding sequence, *OsMTP11.1* expression was not detected in the organs analyzed (Fig. 2). Regarding expression analysis on rice developing stages, *OsMTP11* was highly expressed from first leaf emergence to tillering stage, and from panicle stage 6 to developing seed 1 (Fig. 3). Once more *OsMTP11.1* expression was not detected (Fig. 3).

OsMTP11 in yeast complementation assays

PMR1 encodes a yeast secretory pathway Ca/Mn-ATPase that is located in a Golgi-like compartment (Antebi and Fink, 1992). Yeast *pmr1* deletion strains are Mn-hypersensitive and accumulate Mn to high concentrations (Lapinskas *et al.,* 1995). OsMTP11 partially rescues the mutant phenotype in a range of Mn^{+2} concentrations (0.3 mM, 0.5 mM and 1 mM; Fig. 4). To examine whether OsMTP11 affects homeostasis of yeast with respect to exposure to other metals, we tested for complementation of yeast mutants sensitive to Zn^{+2} (*zrc1 cot1*; Fig. 5) and Co^{+2} (*zrc1 cot1*, Fig. 6). Yeast transformants *zrc1 cot1* were used in tests for Zn tolerance in parallel with Co tolerance. OsMTP11 showed a slight rescue of Zn sensitivity, perhaps with lower affinity (Fig. 5); OsMTP11 did not confer Co tolerance to the *zrc1 cot1* mutant strain (Fig. 6).

OsMTP11 site-directed mutations

The transmembrane data predicted by Phobius was used to construct the hypothetical membrane topology of OsMTP11 in Figure 1A. Four site -directed mutations were tested for their influence on the transport function of OsMTP11 (Table 1). We selected the residues based on conservation between CDF family members (Montanini *et al.,* 2007; Gustin *et al.,* 2011).

Manganese tolerance assay

OsMTP11 partially rescues the *pmr1* mutant phenotype at 0.3, 0.5 and 1 mM of $MnCl_2$. Compared to the *OsMTP11* construct; almost all mutations reduced the rescue of the Mn-sensitive phenotype to some extent. This was obvious in E213G, but most apparent in D267H and D162A, which failed to rescue in the lowest concentration tested; the exception was L150S, which fully rescued *pmr1* Mn-sensitivity in all concentrations tested (Fig. 4).

Zinc tolerance assay

OsMTP11 showed a slight rescue of the *zrc1 cot1* Zn-sensitivity that was perceptible only for the D162A mutation, but not for the other mutations tested (Fig 5).

OsMTP11 complementation of Arabidopsis *mtp11* mutant

Preliminary tests with two heterozygous lines (*atmtp11* expressing *OsMTP11*) showed better growth in vertical plates containing media with 1mM of $MnSO_4$ than the mutant *atmtp11* itself (data not shown). Fresh weight and chlorophyll measurements will be evaluated. Homozygous lines have been selected and complementation is being analyzed.

Discussion

Manganese is an essential micronutrient for plants, playing crucial roles as cofactor of many enzymes. However, the metal is required in very small quantities (Marschner, 1995). Mn toxicity symptoms are caused by excess of Mn^{2+} accumulated in the apoplast, which is oxidized to Mn^{3+}, an ion with strong activity to oxidize proteins and lipids (Fecht-Christoffers et al., 2003a, 2003b). Exposure to elevated levels of Mn, which frequently occurs in acidic soils, causes various toxicity symptoms (Marschner, 1995). Rice is usually cultivated under flooded conditions, where Mn concentration in soil solution is very high due to reduction. Some of the strategies to deal with the excess of this metal at the cellular level are: extrusion of Mn from cytoplasm via plasma membrane transporters; deposition in the vacuole via tonoplast influx transporters (Delhaize *et al.,* 2003; Delhaize *et al.,* 2007; Pittman *et al.,* 2004; Schaaf *et al.,* 2002); and sequestering of the metal into secretory vesicles and subsequent exocytosis via the secretory pathway Ca/Mn-ATPases (Delhaize *et al.,* 2007; Peiter *et al.,* 2007).

CDFs from Group 8 and 9 are important for plant Mn homeostasis. Group 9 shows evidence of a duplication event prior to the monocot/eudicot split. This duplication event produced two Group 9 lineages in higher plants, both of which are maintained, and in some cases expanded (Montanini *et al.,* 2007; Gustin *et al.,* 2011). There are two MTP11 representatives in the rice genome named *OsMTP11* and *OsMTP11.1*. It was not possible to amplify *OsMTP11.1* transcripts from different rice organs even using plants exposed to different metal excess treatments. Expression pattern of *OsMTP11* and *OsMTP11.1* genes based on microarray meta-analysis clearly shows that *OsMTP11.1* expression is either low or not detected in all rice organs and developmental stages analyzed (Figs. 2 and 3). *OsMTP11.1* might be a nonfunctional genomic sequence, a pseudogene; however further studies need to be carried out to further investigate this possibility. *OsMTP11* expression is mainly detected in shoots,

particularly in leaves and panicles, probably being involved in Mn homeostasis, as discussed in the next section (Figs. 2 and 3).

The *OsMTP11* gene complements the Mn-hypersensitive phenotype of *pmr1* yeast mutant cells

Expression of *OsMTP11* in the *pmr1* mutant yeast strain rescues the Mn-sensitive phenotype, suggesting considerable Mn transporting ability of OsMTP11 (Fig. 4). Similarly,expression of *AtMTP11* and *PtMTP11*, from Arabidopsis and poplar, specifically restored Mn tolerance of *pmr1* and both demonstrate to be targeted to endomembranes in a manner similar to the secretory pathway Ca/Mn-ATPase Pmr1p (Delhaize *et al.*, 2007; Peiter *et al.,* 2007). OsMTP11 and AtMTP11 have 78% of amino acid identity and are expected to perform similar functions in plants. Thus, the data suggest that, when heterologously expressed in yeast, *OsMTP11* can substitute *PMR1* function for Mn detoxification in a Golgi-associated compartment. In Arabidopsis, *AtMTP11* overexpression and the *atmtp11* loss of function mutant led to improvement of Mn tolerance and enhanced Mn sensitivity, respectively (Peiter *et al.,* 2007), showing a role of AtMTP11 in Mn homeostasis that is also expected for OsMTP11.

OsMTP11 also achieved partial rescue of *zrc1 cot1* on Zn, in similar levels to HvHMA2 (Fig. 5; Mills *et al.,* 2012) but did not complement the same mutant strain for Co-sensitivity (Fig. 6). Results presented here suggest that OsMTP11 is primarily a Mn transporter but is also capable of transporting Zn. The OsMTP11 substrate specificity is considerably broader than that of the Mn-specific AtMTP11 and PtMTP11.

OsMTP11 amino acid residues acting in transport activity

There isn't information in the literature about metal-binding domains and metal coordination sites for proteins from the Mn-CDFs group. We chose residues widely conserved between Mn-CDFs and Zn-CDFs for the site-directed mutagenesis experiments, aiming to catch important residues involved in substrate specificity and transport.

We used the yeast system to study the functional significance of key residues in OsMTP11. The Mn-CDF sequences could be differentiated by the consensus sequence DxxxD (x = any amino acid) in TMD V, which appears as HxxxD in all other CDF sequences from the Zn- and Fe/Zn-CDFs, D being the highly conserved aspartate residue important for CDF function (Montanini *et al.,* 2007). Substitution of aspartate 267 for histidine (D267H) entirely abolishes Mn transport function of OsMTP11 in the lowest concentration tested (0.3 mM; Fig. 4); and even abolished Zn transport (Fig. 5), that was expected to improve as the histidine is conserved

in the Zn-CDF group. Since the group-conserved H/D residue is four residues distant from the highly conserved aspartate within TMD V, the two residues will interact on the same face of the **α**-helix. These residues may play important but different roles in the CDF groups (Montanini et al., 2007).

The L150S mutation falls in the first non-cytoplasmic loop at the beginning of the CDF signature sequence (Blaudez *et al.*, 2003; Montanini et al, 2007). This mutation fully rescued the *pmr1* Mn-sensitivity, being more effective on Mn transport than the wt protein (Fig. 4); and abolished Zn transport (Fig. 5). It is homologous to the L33F mutation in ScZRC1, which reduced Zn transport but increased Fe and Mn affinity (Lin *et al.*, 2009). It may, therefore, be concluded that L150 is an important residue for metal selectivity in plant CDFs as well as in yeast transporters. D162A also falls in the CDF signature (Blaudez *et al.*, 2003; Montanini et al, 2007), abolished Mn transport (Fig. 4) and reduced Zn transport (Fig. 5). The homologous mutation in AtMTP1 lost Zn transport activity completely. This indicates that these residues are required for metal transport (Kawachi *et al.*, 2012).

E213G is homologous to E97G, a mutation in ScZRC1 that completely shifts the transported substrate from Zn to Fe and Mn (Lin *et al.*, 2009). Contrastingly, the corresponding AtMTP1 mutant just extends its transport ability to Co and Mn (Podar *et al.*, 2012) and when mutated to the non polar alanine, E145A, extends its transport to Co and Cd (Kawachi *et al.*, 2012). In contrast to the results with ScZRC1, OsMTP11-E213G continues to transport Mn, at a slightly lower level than the non-mutated construct, and abolished Zn transport. E145 is a polar residue falling within a region containing conserved polar residues thought to be involved in metal transport across the family of CDF proteins (Gaither and Eide, 2001; Haney *et al.*, 2005). It may, therefore, be a further important residue for the specificity of OsMTP11. Further studies using different techniques such as OsMTP11 random-mutagenesis will be important to discover specific residues acting in Mn-CDFs activity.

Conclusion

There are two MTP11 sequences in the rice genome, *OsMTP11* and *OsMTP11.1*, the last being probably a pseudogene, as gene expression is low or not detected. *OsMTP11* achieves higher expression levels in shoots and the protein appears to transport Mn and Zn, possibly with lower affinity. L150S, D162, E213 and D267 seem to be important residues for OsMTP11 transport activity. These are the first findings on OsMTP11 characterization described in the literature.

Acknowledgements

We gratefully acknowledge Prof. Ute Kramer (Ruhr University, Bochum, Germany) for the *zrc1cot1* mutant yeast strain. This research was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil), research fellowship to JPF and scholarships to FKR and PKM; by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil), sandwich scholarship to PKM; and partly by funding to LEW from the European Union Framework Programme 6 as part of the Integrated Project on Public Health Impact of long-term, low level Mixed element Exposure in susceptible population strata (PHIME) (contract number FOOD-CT-2006- 016253); it reflects only the authors' views. The Community is not liable for any use that may be made of the information contained therein. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

Antebi A, Fink GR (1992) The yeast Ca2+-ATPase homologue, *PMR1*, is required for normal Golgi function and localizes in a novel Golgi-like distribution. Mol Biol Cell 3: 633–654.

Blaudez D, Kohler A, Martin F, Sanders D, Chalot M (2003) Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. Plant Cell 15: 2911–2928.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743.

Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR (2003) Plant Cell 15:1131–1142.

Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardon AE (2007) A role for the *AtMTP11* gene of *Arabidopsis* in manganese transport and tolerance. The Plant J 51: 198–210.

Fecht-Christoffers, MM, Braun HP, Lemaitre-Guillier C, Horst, WJ (2003a) Effect of manganese toxicity on the proteome of the leaf apoplast in Cowpea. Plant Physiol 133: 1935–1946.

Fecht-Christoffers, MM, Maier P, Horst WJ (2003b) Apoplastic peroxidases and ascorbate are involved in manganese toxicity and tolerance of *Vigna unguiculata.* Physiol Plant 117: 237-244.

Gaither, L.A. and Eide, D.J. (2001) Eukaryotic zinc transporters and their regulation. Biometals 14, 251–270.

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acid Res 20: 1425.

Gustin JL, Zanis MJ, Salt DE (2011) Structure and evolution of the plant cation diffusion facilitator family of ion transporters. BMC Evol Biol 11: 76.

Haney CJ, Grass G, Franke S, Rensing C (2005) New developments in the understanding of the cation diffusion facilitator family. J Ind Microb Biot 32: 215-226.

Kawachi M, Kobae Y, Kogawa S, Mimura T, Kramer U, Maeshima M (2012) Amino acid screening based on structural modeling identifies critical residues for function, ion selectivity and structure of Arabidopsis MTP1. FEBS J 279: 2339-2356.

Kurup S, Runions J, Köhler U, Laplaze L, Hodge S, Haseloff J (2005) Marking cell lineages in living tissues. Plant J 42: 444–453.

Lapinskas PJ, Cunningham KW, Liu XF, Fink GR, Culotta VC (1995) Mutations in *PMR1* suppress oxidative damage in yeast cells lacking superoxide dismutase. Mol Cell Biol 15:1382–1388.

Lin H, Burton D, Li L, Warner DE, Phillips JD, Ward DM, Kaplan J (2009) Gain-of-function mutations identify amino acids within transmembrane domains of the yeast vacuolar transporter Zrc1 that determine metal specificity. Biochem J 422: 273–283.

Marschner H (1995) Mineral nutrition of higher plants. 2 ed. Academic Press, New York.

Mills RF, Doherty ML, Lopez-Marques RL, Weimar T, Dupree P, Palmgren MG, Pittman JK, Williams LE (2008) ECA3, a Golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in Arabidopsis. Plant Physiol 146: 116-128.

Mills RF, Valdes B, Duke M, Peaston KA, Lahner B, Salt DE, Williams LE (2010) Functional significance of AtHMA4 C-terminal domain in planta. PLoS One 5, e13388.

Mills RF, Peaston KA, Runions J, Williams LE. 2012. HvHMA2, a P(1B)-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. PLoS One 7, e42640.

Montanini B, Blaudez D, Jeandroz S, Sanders D, Chalot M (2007) Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. BMC Genomics 8: 107.

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using n,n-dimethylformamide. Plant Physiol 65: 478-479.

Murashige T, Skoog FA (1962) A revised medium for a rapid growth and bioassays with tobacco tissues cultures. Plant Physiol 15: 473-479.

Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D (2007) A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. Plant Biol 104: 8532–8537.

Pittman JK, Shigaki T, Marshall JL, Morris JL, Cheng NH, Hirschi KD (2004) Functional and regulatory analysis of the Arabidopsis thaliana CAX2 cation transporter. Plant Mol Biol 56: 959–971.

Podar D, Scherer J, Noordally Z, Herzyk P, Nies D, Sanders D (2012) Metal selectivity determinants in a family of transition metal transporters. J Biol Chem 287: 3185-3196.

Schaaf G, Catoni E, Fitz M, Schwacke R, Schneider A, von Wirén N, Frommer WB (2002) A putative role for the vacuolar calcium/manganese proton antiporter AtCAX2 in heavy metal detoxification. Plant Biol 4: 612–618.

Tables

Table 1: Substitutions resulting from site directed mutations of OsMTP11

Position	TMDII	TMDII	IL2	EL2
Substitution	L150S	D162A	E213G	D267H

Figure Legends:

Figure 1: A, hypothetical membrane topology of OsMTP11 predicted according to the Phobius program. B, hypothetical membrane topology of OsMTP11.1 predicted according to the Phobius program. EL, extracytosolic loop; IL, intracytosolic loop.

Figure 2: *OsMTP11* (LOC_Os01g62070) and *OsMTP11.1* (LOC_Os05g38670) gene expression data obtained using the Rice Oligonucleotide Array Database, and based on Affymetrix specific probes. All available high quality arrays on rice organ-specific expression were used. Expression level is denoted by intensity of yellow color. Organ names are given above.

Figure 3: *OsMTP11* (LOC_Os01g62070) and *OsMTP11.1* (LOC_Os05g38670) gene expression data obtained using the Rice Oligonucleotide Array Database, and based on Affymetrix specific probes. All available high quality arrays on rice developing stages expression were used. Expression level is denoted by intensity of yellow color. Developing stages are given above.

Figure 4: Functional complementation of manganese-sensitive *Saccharomyces cerevisiae* mutant *pmr1*. Yeast strain K601 was transformed with pYTV empty vector (e.v.); *pmr1* was transformed with pYTV vector either empty or containing *OsMTP11* full length. L150S to E213G refer to site directed substitutions generated within *OsMTP11*. Numbers represent serial dilutions of yeast cells in in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with 0, 0.3, 0.5 and 1 mM of $MnCl_2$. Plates were incubated at 28°C for 5 days.

Figure 5: Functional analysis of *OsMTP11* in zinc-sensitive *Saccharomyces cerevisiae* mutant *zrc1cot1*. Yeast strain BY4741 was transformed with pYTV empty vector (e.v.); z*rc1 cot1* was transformed with pYTV vector either empty or expressing *OsMTP11* full length; HvHMA2 was used as a positive control. Numbers represent serial dilutions of yeast cells in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with 0.3 mM of $ZnSO_4$. Plates were incubated at 28°C for 5 days.

Figure 6: Functional analysis of *OsMTP11* in cobalt-sensitive *Saccharomyces cerevisiae* mutant *zrc1cot1*. Yeast strain BY4741 was transformed with pYTV empty vector (e.v.); z*rc1 cot1* was transformed with pYTV vector either empty or expressing *OsMTP11* full length; OsMTP1 was used as a positive control. Numbers represent serial dilutions of yeast cells in liquid SC galactose without uracil: undiluted (1) OD_{600}=0.4, 1:10 and 1:100; sequentially dropped onto and grown on SC galactose uracil-dropout media with 0, 0.2, 0.5, 1 mM of $CoCl_2$. Plates were incubated at 28°C for 5 days.

Figure 1:

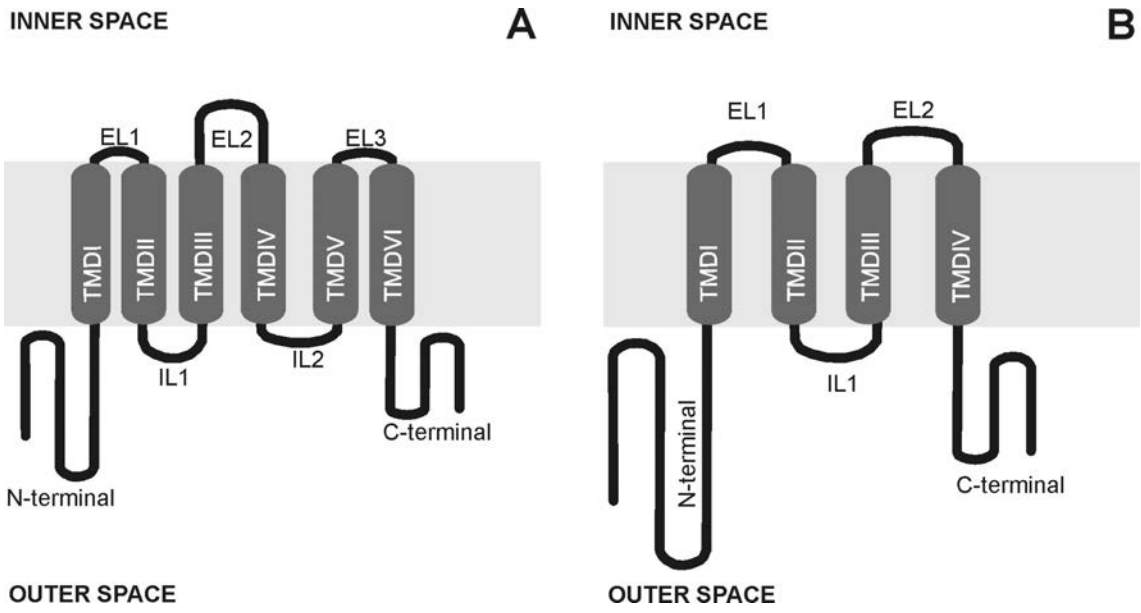

INNER SPACE
A
EL1
EL2
EL3
TMDI
TMDII
TMDIII
TMDIV
TMDV
TMDVI
IL1
IL2
C-terminal
N-terminal
OUTER SPACE
INNER SPACE
B
EL1
EL2
TMDI
TMDII
TMDIII
TMDIV
IL1
N-terminal
C-terminal
OUTER SPACE

Figure 2:

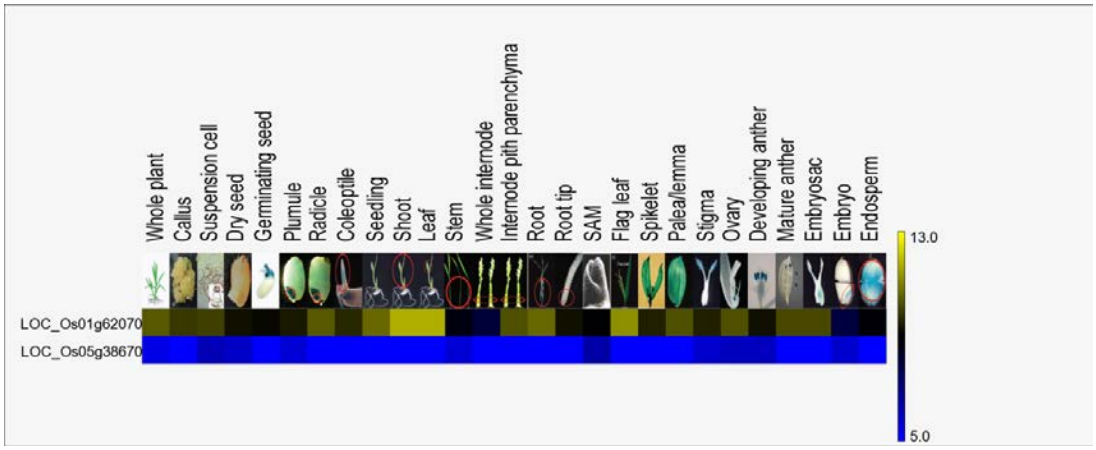

Whole plant
Callus
Suspension cell
Dry seed
Germinating seed
Plumule
Radicle
Coleoptile
Seedling
Shoot
Leaf
Stem
Whole internode
Internode pith parenchyma
Root
Root tip
SAM
Flag leaf
Spikelet
Palea/lemma
Stigma
Ovary
Developing anther
Mature anther
Embryosac
Embryo
Endosperm
LOC_Os01g62070
LOC_Os05g38670
13.0
5.0

Figure 3:

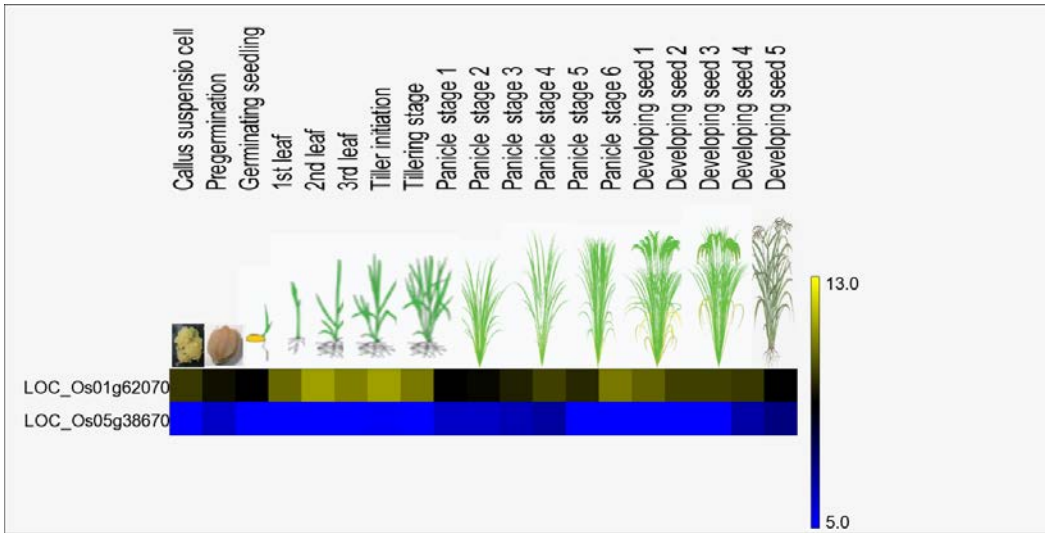

Callus suspensio cell
Pregermination
Germinating seedling
1st leaf
2nd leaf
3rd leaf
Tiller initiation
Tillering stage
Panicle stage 1
Panicle stage 2
Panicle stage 3
Panicle stage 4
Panicle stage 5
Panicle stage 6
Developing seed 1
Developing seed 2
Developing seed 3
Developing seed 4
Developing seed 5
LOC_Os01g62070
LOC_Os05g38670
13.0
5.0

Figure 4:

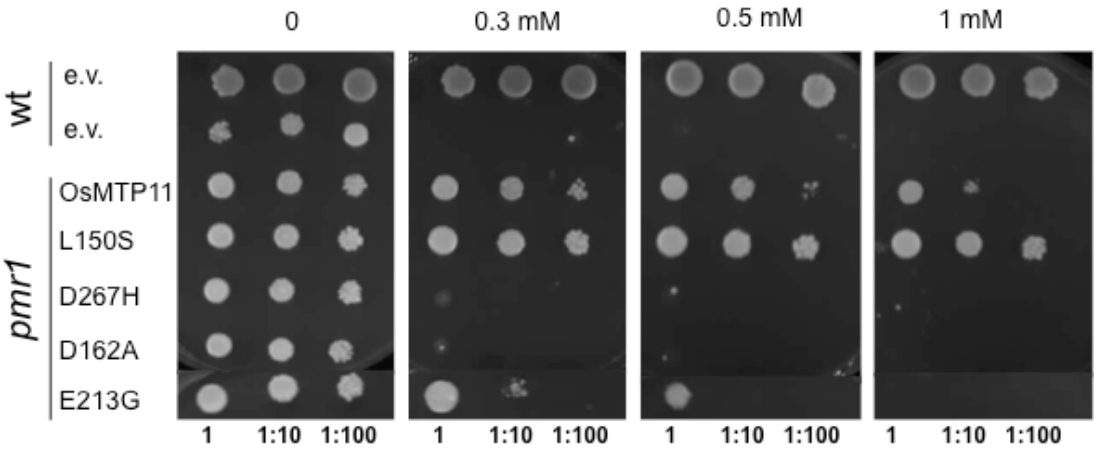

0
0.3 mM
0.5 mM
1 mM
wt
e.v.
e.v.
pmr1
OsMTP11
L150S
D267H
D162A
E213G
1
1:10
1:100
1
1:10
1:100
1
1:10
1:100
1
1:10
1:100

Figure 5:

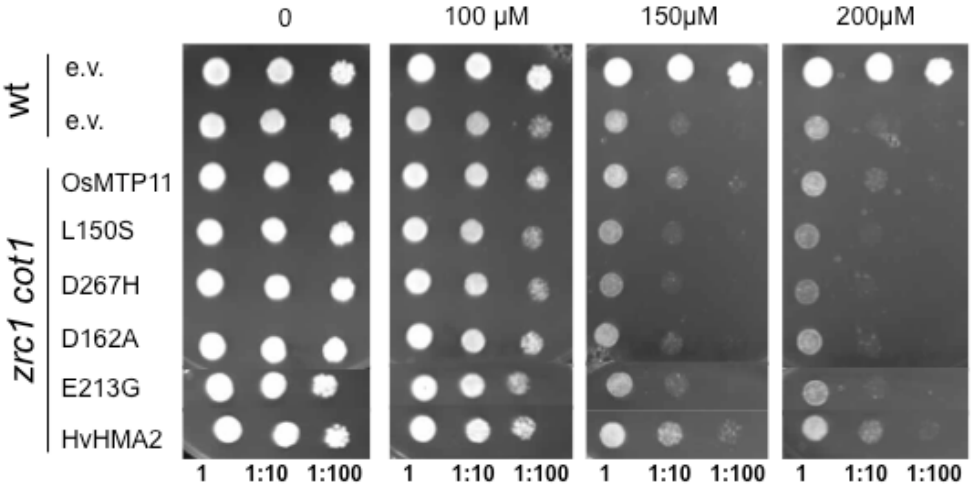
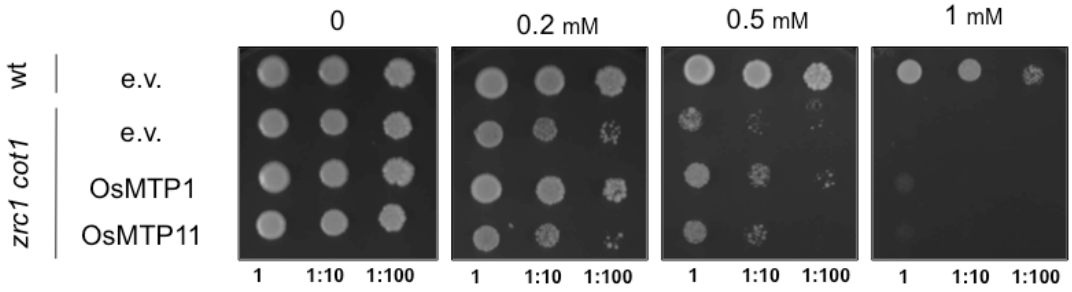

0
100 µM
150µM
200µM
wt
e.v.
e.v.
zrc1 cot1
OsMTP11
L150S
D267H
D162A
E213G
HvHMA2
1
1:10
1:100

Figure 6:



Molecular characterization of a rice metal tolerance protein OsMTP11

Paloma K. Menguer, Kerry A. Peaston, Felipe Klein Ricachenevsky, Raul Antônio Sperotto, Janette Palma Fett, Lorraine E. Williams

Supplementary material

Table S1: List of primers used for cloning OsMTP11 site-directed mutations.

Name	Sequence
OsMTP11 L150S_F	GTGTATGCTTCTGTGAGAAGTGGTTCAAGTGCTATTATTGCTTCGAC
OsMTP11 L150S_R	GTCGAAGCAATAATAGCACTTGAACCACTTCTCACAGAAGCATACAC
OsMTP11 D162A_F	CGACTTTGGATTCTCTTCTTGCCTTGTTGTCAGGATTTATCTT
OsMTP11 D162A_R	AAGATAAATCCTGACAACAAGGCAAGAAGAGAATCCAAAGTCG
OsMTP11 E213G_F	CCTTCAGATCATCCTAGGATCAGTACGCTCGTTGT
OsMTP11 E213G_R	ACAACGAGCGTACTGATCCTAGGATGATCTGAAGG
OsMTP11 D267H_F	ATAGTGAAGGCTTATGCACAGCATCATTTTTTTGATGTCATCAC
OsMTP11 D267H_R	GTGATGACATCAAAAAAATGATGCTGTGCATAAGCCTTCACTAT

Table S2: Affimetrix probes (main probes are the ones with highest expression and were used in figures 2 and 3.

Gene ID	Main Probeset	Other Probesets	Gene Annotation
LOC_Os01g62070	Os.10121.1.S1_at	Os.42490.1.S1_at Os.42490.1.S1_x_at	putative, expressed
LOC_Os05g38670	Os.10121.1.S1_at	-	putative

Considerações Finais

OsMTP1 é um transportador vacuolar de Zn com baixa afinidade de tranporte para outros metais

Utilizando diversas abordagens foi possível concluir que OsMTP1 é um transportador vacuolar de Zn, que também é capaz de transportar outros metais como Co, Fe e Cd. AtMTP1 é um transportador específico de Zn e AtMTP3 pode transportar Zn e Co; da mesma maneira HvMTP1 também possui um transporte mais amplo de metais, incluíndo Zn e Co (Kobae *et al.*, 2004; Desbrosses-Fonrouge *et al.*, 2005; Arrivault *et al.*, 2006; Podar *et al.*, 2012). Filogeneticamente, o grupo 1 dos MTPs passou por diversos eventos de duplicação durante a evolução; seus membros são MTP1, MTP2 (o cluster de MTP1 e MTP2 é referido como MTP1/2), MTP3 e MTP4 (Gustin *et al.*, 2011). Das monocotiledôneas que foram analisadas até o momento, incluindo arroz, não possuem a sequência de *MTP4* no seu genoma, sugerindo que as mesmas possam ter perdido este gene. Análises filogenéticas indicam que as sequências *MTP1/2* e *MTP3* possuem um ancestral comum; quando ocorreu a duplicação, algum tempo depois da divergência entre monocotiledôneas e eudicotiledôneas, MTP1/2 e MTP3 provavelmente partilharam funções redundantes (Gustin *et al.*, 2011). Enquanto as funções de MTP1/2 e MTP3 divergiram em Arabidopsis, em arroz MTP1 pode ter mantido o funcionamento do gene ancestral, já que esta espécie não possui as sequências de *MTP2* e *MTP3* no seu genoma (Gustin *et al.*, 2011); o que pode explicar o transporte mais amplo de metais em OsMTP1.

Estudos de mutação sítio-dirigida revelaram duas substituções que podem ser importantes na determinação da especificidade de substrato em OsMTP1. Substituindo leucina 82 por fenilalanina OsMTP1 ainda transporta baixos níveis de Zn, com aumento da afinidade por Fe e Co, e também começa a transportar Mn. Na substituição de histidina 90 por ácido aspártico, abole-se o transporte de Zn, mas o transporte de Fe é aumentado. Este é o primeiro trabalho visando a identificação de resíduos envolvidos na especificidade e transporte de metais em OsMTP1.

Caracterização de OsMTP11

Este é o primeiro trabalho presente na literatura caracterizando OsMTP11, homólogo de AtMTP11, é esperado o envolvimento dessa proteína no transporte de Mn para dentro de compartimentos pré-vacuolares ou trans-golgi. Com base nos experimentos realizados é possível sugerir que OsMTP11 é um transportador de Mn, também capaz de transportar Zn com baixa afinidade. Estudos de mutação sítio-dirigida identificaram quatro resíduos envolvidos na função de transporte em OsMTP11; com especial ênfase para a substituição da leucina 150 por serina, que aumenta significativamente a complementação da levedura mutante *pmr1* (hipersensível a Mn). Experimentos de complementação do mutante de Arabidopsis *mtp11* pela superexpressão de *OsMTP11* e de localização da proteína em sistema de expressão estável com fusão a GFP estão sendo conduzidos para uma melhor caracterização desse transportador.

Referências Gerais

Alberti S, Gitler AD, Lindquist S (2007) A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. Yeast 24: 913-919

Andreini C, Bertini I, Cavallaro G, Holliday GL, Thornton JM (2008) Metal ions in biological catalysis: From enzyme databases to general principles. J Biol Inorg Chem 13:1205–1218

Antebi A, Fink GR (1992) The yeast Ca2+-ATPase homologue, *PMR1*, is required for normal Golgi function and localizes in a novel Golgi-like distribution. Mol Biol Cell 3: 633–654

Arrivault S, Senger T, Krämer U (2006) The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. Plant J 46: 861–879

Assunção AG, Herrero E, Lin YF, Huettel B, Talukdar S, Smaczniak C, Immink RG, van Eldik M, Fiers M, Schat H, Aarts MG (2010) *Arabidopsis thaliana* transcription factors bZIP19 and bZIP23 regulate the adaptation to zinc deficiency. Proc Natl Acad Sci USA 107: 10296–10301.

Baekgaard L, Mikkelsen MD, Sorensen DM, Hegelund JN, Persson DP, Mills RF, Yang Z, Husted S, Andersen JP, Buch-Pedersen MJ, Schjoerring JK, Williams LE, Palmgren MG (2010) A combined zinc/cadmium sensor and zinc/cadmium export regulator in a heavy metal pump. J Biol Chem 285: 31243-31252.

Becher M, Talke IN, Krall L, Krämer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37: 251–268.

Berg JM, Shi Y (1996) The galvanization of biology: a growing appre- ciation for the roles of zinc. Science 271:1081–1085.

Bergmann W (1992) Nutritional Disorders of Plants (Gustav Fischer, Jena, Germany).

Blamey FPC, Joyce DC, Edwards DG, Asher CJ (1986) Plant Soil 91:171–180.

Blaudez D, Kohler A, Martin F, Sanders D, Chalot M (2003) Poplar metal tolerance protein 1 confers zinc tolerance and is an oligomeric vacuolar zinc transporter with an essential leucine zipper motif. Plant Cell 15: 2911–2928.

Bloss T, Clemens S, Nies D (2002) Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomes. Planta 214: 783–791.

Bouis HE, Welch RM (2010) Biofortification - a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. Crop Sci 50: S20-S32.

Bregitzer PP, Raboy V (2006) Effects of four independent low-phytate mutations on barley (*Hordeum vulgare* L.) agronomic performances. Crop Sci 46: 1318-1322.

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. New Phytol 173: 677-702.

Cakmak I, Tolay I, Ozdemir A, Ozkan H, Kling CI (1999) Differences in zinc efficiency among and within diploid, tetraploid and hexaploid wheats. Ann Bot 84: 163-71.

Cakmak I, Ozkan H, Braun HJ, Welch RM, Romheld V (2000) Zinc and iron concentrations in seeds of wild, primitive and modern wheats. Food Nutr Bull 21: 401-403.

Chao Y, Fu D (2004) Kinetic study of the antiport mechanism of an *Escherichia coli* zinc transporter, ZitB. J Biol Chem 279: 12043–12050

Cherezov V, Hofer N, Szebenyi DM, Kolaj O, Wall JG, Gillilan R, Srinivasan V, Jaroniec CP, Caffrey M (2008) Insights into the mode of action of a putative zinc transporter CzrB in *Thermus thermophilus*. Struct 16: 1378-1388.

Clarkson DT (1988) The uptake and translocation of manganese by plant roots. In: Graham RD, Hannam RJ, Uren NC, eds. Manganese in soils and plants. Dordrecht, the Netherlands: Kluwer Academic Publishers, 101–111

Clemens, S (2001) Molecular mechanisms of plant metal tolerance and homeosta- sis. Planta 212: 475–486

Clemens S, Palmgren MG, Krämer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends Plant Sci 7: 309-315

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743.

Conklin DS, Culbertson MR, Kung C (1994) Interactions between gene products involved in divalent cation transport in *Saccharomyces cerevisiae*. Mol Gen Genet. 244: 303–311

Conklin, D.S., McMaster, J.A., Culbertson, M.R. and Kung, C. (1992) *COT1*, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*. Mol Cell Biol 12: 3678–3688

Conn SJ, Berninger P, Broadley MR, Gilliham M (2012) Exploiting natural variation to uncover candidate genes that control element accumulation in *Arabidopsis thaliana*. New Phytol 193: 859–866

Connolly EL, Fett JP, Guerinot ML (2002) Expression of the *IRT1* metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14: 1347–1357

Cragg RA, Christie GR, Phillips SR, Russi RM, Küry S, Mathers JC, Taylor PM, Ford D (2002) A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. J Biol Chem 277: 22789–22797

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Phys 133: 462-469

Drager DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, et al (2004) Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. Plant J 39: 425–439

Delhaize E, Kataoka T, Hebb DM, White RG, Ryan PR (2003) Plant Cell 15:1131–1142

Delhaize E, Gruber BD, Pittman JK, White RG, Leung H, Miao Y, Jiang L, Ryan PR, Richardon AE (2007) A role for the *AtMTP11* gene of *Arabidopsis* in manganese transport and tolerance. The Plant J 51:198–210

Desbrosses-Fonrouge AG, Voigt K, Schröder A, Arrivault S, Thomine S, Krämer U (2005) *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and

drives leaf Zn accumulation. FEBS Lett 579: 4165–4174

Eckhardt U, Marques AM, Buckhout TJ (2001) Two iron-regulated cation transporters from tomato complement metal uptake-deficient yeast mutants. Plant Mol Biol 45: 437–448

Eide D, Broderius M, Fett J, Guerinot ML (1996) A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc Natl Acad Sci USA 93: 5624–5628.
El-Jaoual T, Cox DA (1998) J Plant Nutr 21:353–386

Fecht-Christoffers, MM, Braun HP, Lemaitre-Guillier C, Horst, WJ (2003a) Effect of manganese toxicity on the proteome of the leaf apoplast in Cowpea. Plant Physiol 133: 1935–1946.

Fecht-Christoffers, MM, Maier P, Horst WJ (2003b) Apoplastic peroxidases and ascorbate are involved in manganese toxicity and tolerance of *Vigna unguiculata*. Physiol Plant 117: 237-244.

Fraústo da Silva JJR, Williams RJP (2001) The Biological Chemistry of the Elements, Ed 2. Oxford University Press, New York

Gaither, L.A. and Eide, D.J. (2000) Functional expression of the human hZIP2 zinc transporter. J Biol Chem 275, 5560–5564

Gaither, L.A. and Eide, D.J. (2001) Eukaryotic zinc transporters and their regulation. Biometals 14, 251–270

Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20: 1425.

Gitan RS, Luo H, Rodgers J, Broderius M, Eide DJ (1998) Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J Biol Chem 273: 28617–28624

Goff SA, Ricke D, Lan TH, Presting G, Wang R, Dunn M, Glazebrook J, et al (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. japonica). Science 296: 92-100

Gómez-Galera S, Rojas E, Sudhakar D, Zhu C, Pelacho AM, Capell T, Christou P (2010) Critical evaluation of strategies for mineral fortification of staple food crops. Transgenic Research 19: 165-180

González A, Lynch JP (1999) Aust J Plant Physiol 26:811–822

Graham RD and Rengel Z (1993) Genotypic variation in zinc uptake and utilization by plants. In: Robson AD (ed.) Zinc in Soils and Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands pp.107–118

Graham RD, Senadhira D, Beebe SE, Iglesias C, Ortiz-Monasterio I (1999) Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. Field Crop Res 60: 57-80

Grass G, Otto M, Fricke B, Haney CJ, Rensing C, Nies DH, Munkelt D (2005) FieF (YiiP) from *Escherichia coli* mediates decreased cellular accumulation of iron and relieves iron stress. Arch Microbiol 183, 9 –18

Gregorio G, Senadhira D, Htut H, Graham RD (2000) Breeding for trace mineral density in rice. Food Nutr Bull 21: 382-386

Grotz N, Fox T, Connolly E, Park W, Guerinot ML, Eide D (1998) Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proc Natl Acad Sci USA 95: 7220–7224

Grotz N and Guerinot ML (2006) Molecular aspects of Cu, Fe and Zn homeostasis in plants. Biochim Biophys Acta 1763, 595–608

Guffanti AA, Wei Y, Rood SV, Krulwich TA (2002) An antiport mechanism for a member of the cation diffusion facilitator family: divalent cations efflux in exchange for K^+ and H^+. Mol Microbiol 45: 145–53

Gustin JL, Loureiro ME, Kim D, Na G, Tikhonova M, Salt DE (2009) MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. Plant J 57: 1116-1127

Gustin JL, Zanis MJ, Salt DE (2011) Structure and evolution of the plant cation diffusion facilitator family of ion transporters. BMC Evol Biol 11: 76

Hall JL, Williams LE (2003) Transition metal transporters in plants. J Exp Bot 54: 2601-2613.

Hambidge KM, Huffer JW, Raboy V, et al (2004) Zinc absorption from low- phytate hybrids of maize and their wild-type isohybrids. Am J Clin Nutr 79:1053–9

Haney CJ, Grass G, Franke S, Rensing C (2005) New developments in the understanding of the cation diffusion facilitator family. J Ind Microbiol Biotech 32: 215-226.

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Kramer U (2008) Evolution of metal hyperaccumulation required *cis*-regulatory changes and triplication of *HMA4*. Nature 453: 391-395

Hanikenne M, Nouet C (2011) Metal hyperaccumulation and hypertolerance: a model for plant evolutionary genomics. Curr Opin Plant Biol 14: 252-259.

HarvestPlus (2010) HarvestPlus Statement on the Potential Benefits of Biofortification on the Nutritional Status of Populations. Available at http://www.harvestplus.org. Acessed 08 Jan 2013

Haydon MJ, Cobbett CS (2007) A novel major facilitator superfamily protein at the tonoplast influences zinc tolerance and accumulation in Arabidopsis. Plant Physiol 143: 1705–1719

Hegelund JN, Schiller M, Kichey T, Hansen TH, Pedas P, Husted S, Schjoerring JK (2012) Barley Metallothioneins: MT3 and MT4 Are Localized in the Grain Aleurone Layer and Show Differential Zinc Binding. Plant Physiol 159: 1125–1137

Hirano T, Murakami M, Fukada T, Nishida K, Yamasaki S, Suzuki T (2008) Roles of zinc and zinc signaling in immunity: zinc as an intracellular signaling molecule. Advances in Immunology 97: 149-176

Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000) Plant Physiol 124:125–133

Hoch E, Lin W, Chai J, Hershfinkel M, Fu D, Sekler I (2012) Histidine pairing at the metal transport site of mammalian ZnT transporters controls Zn2+ over Cd2+ selectivity. Proc Natl Acad Sci USA 109: 7202–7207

Hu H, Sparks D, Evans JJ (1991) Sulfur deficiency influences vegetative growth, chlorophyll and element concentrations, and amino acids of pecan. J Amer Soc Hort Sci 116: 974-980

IBGE - Instituto Brasileiro de Geografia e Estatística. Levantamento Sistemático da Produção Agrícola. Disponível em: http://www.ibge.gov.br/home/estatistica/indicadores/agropecuaria/lspa/default_publ_completa.shtm

IRGSP - International Rice Genome Sequencing Project. (2005) The map-based sequence of the rice genome. Nature 436: 793-800

IRRI – International Rice Research Institute. Accessed in December 8th, 2012. Available at: http://www.irri.org/

Ishimaru Y, Suzuki M, Kobayashi T, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2005) OsZIP4, a novel zinc-regulated zinc transporter in rice. J Exp Bot 56: 3207–3214

Ishimaru Y, Kim S, Tsukamoto T, Oki H, Kobayashi T, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2007) Mutational reconstructed ferric chelate reductase confers enhanced tolerance in rice to iron deficiency in calcareous soil Proc Natl Acad Sci USA 104: 7373–7378

Jakubovics NS, Jenkinson HF (2001) Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. Microbiology 147: 1709–1718

Johnson AAT, Kyriacou B, Callahan DL, Carruthers L, Stangoulis J, Lombi E, Tester M (2011) Constitutive overexpression of the *OsNAS* gene family reveals single-gene strategies for effective Iron- and Zinc-biofortification of rice endosperm. PLoS ONE 6: e24476

Kall L, Krogh A, Sonnhammer EL (2004) A combined transmembrane topology and signal peptide prediction method. J Mol Biol 338: 1027-1036

Kamizono A, Nishizawa M, Teranishi Y, Murata K, Kimura A (1989) Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. Mol Gen Genet 219: 161–167

Kawachi M, Kobae Y, Mimura T, Maeshima M (2008) Deletion of a histidine-rich loop of AtMTP1, a vacuolar Zn(2+)/H(+) antiporter of *Arabidopsis thaliana*, stimulates the transport activity. J Biol Chem 283: 8374–8383

Kawachi M, Kobae Y, Mori H, Tomioka R, Lee Y, Maeshima M (2009) A mutant strain *Arabidopsis thaliana* that lacks vacuolar membrane zinc transporter MTP1 revealed the latent tolerance to excessive zinc. Plant Cell Physiol 50: 1156–1170.

Kawachi M, Kobae Y, Kogawa S, Mimura T, Kramer U, Maeshima M (2012) Amino acid screening based on structural modeling identifies critical residues for function, ion selectivity and structure of Arabidopsis MTP1. FEBS J 279: 2339-2356.

Kennedy G, Burlingame B (2003) Analysis of food composition data on rice from a plant genetic resources perspective. Food Chemistry 80: 589–596

Kim BE, Wang F, Dufner-Beattie J, Andrews GK, Eide DJ, Petris MJ (2003) Zn2+-stimulated endocytosis of the mZIP4 zinc transporter regulates its location at the plasma membrane. J Biol Chem 279: 4523–4530

Kim D, Gustin JL, Lahner B, Persans MW, Baek D, Yun DJ, Salt DE (2004) The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. Plant J 39: 237–251

Kim SA, Punshon T, Lanzirotti A, Li L, Alonso JM, Ecker JR, Kaplan J, Guerinot ML (2006) Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1. Science 314: 1295-1298

Kobae Y, Uemura T, Sato MH, Ohnishi M, Mimura T, Nakagawa T, Maeshima M (2004) Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant Cell Physiol 45: 1749–1758

Kobayashi T, Suzuki M, Inoue H, Itai RN, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2005) Expression of iron-acquisition-related genes in iron-deficient rice is coordinately induced by partially conserved iron-deficiency-responsive elements. J Exp Bot 56: 1305–1316

Koike S, Inoue H, Mizuno D, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2004) OsYSL2 is a rice metal-nicotianamine transporter that is regulated by iron and expressed in the phloem. Plant J 39: 415–424

Korshunova YO, Eide D, Clark WG, Guerinot ML, Pakrasi HB (1999) The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Mol Biol 40: 37–44

Krämer U and Clemens S (2005) Functions and homeostasis of zinc, copper, and nickel in plants. In: Tama´s M, Martinoia E (eds) Molecular Biology of Metal Homeostasis and Detoxification. Springer, Berlin, pp 216–271

Krogh A, Larsson B, von Heijne G, Sonnhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305: 567-580.

Kurup S, Runions J, Köhler U, Laplaze L, Hodge S, Haseloff J (2005) Marking cell lineages in living tissues. Plant J 42: 444–453

Lan HX, Wang ZF, Wang QH, Wang MM, Bao YM, Huang J, Zhang HS (2012) Characterization of a vacuolar zinc transporter OZT1 in rice (*Oryza sativa* L.). Mol Biol Rep 40: 1201-1210.

Lanquar V, Lelièvre F, Bolte S, Hamès C, Alcon C, Neumann D, Vansuyt G, Curie C, Schröder A, Krämer U, Barbier-Brygoo H, Thomine S (2005) Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. EMBO J 24: 4041–4051

Lapinskas PJ, Cunningham KW, Liu XF, Fink GR, Culotta VC (1995) Mutations in *PMR1* suppress oxidative damage in yeast cells lacking superoxide dismutase. Mol Cell Biol 15:1382–1388.

Laurie SH, Tancock NP, McGrath SP, Sanders JR (1995) Influence of EDTA complexation on plant uptake of manganese (II). Plant Science 109: 231–235

Lee S, Jeon US, Lee SJ, Kim YK, Persson DP, Husted S, Schjørring JK, Kakei Y, Masuda H, Nishizawa NK, An G (2009) Iron fortification of rice seeds through activation of the nicotianamine synthase gene. Proc Natl Acad Sci USA 106: 22014-22019

Lee S, Kim SA, Lee J, Guerinot ML, An G (2010a) Zinc deficiency-inducible OsZIP8 encodes a plasma membrane-localized zinc transporter in rice. Mol Cells 29: 551-8

Lee S, Jeong HJ, Kim SA, Lee J, Guerinot ML, An G (2010b) OsZIP5 is a plasma membrane zinc transporter in rice. Plant Mol Biol 73: 507-17

Lee S, Persson DP, Hansen TH, Husted S, Schjoerring JK, Kim YS, Kakei Y, Masuda H, Nishizawa NK, An G (2011) Bio-available zinc in rice seeds is increased by activation tagging of nicotianamine synthase. Plant Biotechnol J 9: 865-873

Lin H, Burton D, Li L, Warner DE, Phillips JD, Ward DM, Kaplan J (2009) Gain-of-function mutations identify amino acids within transmembrane domains of the yeast vacuolar transporter Zrc1 that determine metal specificity. Biochem J 422: 273–283

Liu QL, Xu XH, Ren XL, Fu HW, Wu DX, Shu QY (2007) Generation and characterization of low phytic acid germplasm in rice (*Oryza sativa* L.). Theor Appl Genet 114: 803-814

Lönnerdal B, Mendoza C, Brown KH, Rutger JN, Raboy V (2011) Zinc absorption from low phytic acid genotypes of maize (*Zea mays* L.), Barley (*Hordeum vulgare* L.), and Rice (*Oryza sativa* L.) assessed in a suckling rat pup model. J Agr Food Chem 59: 4755-4762

López-Millán AF, Ellis DR, Grusak MA (2004) Identification and characterization of several new members of the ZIP family of metal ion transporters in Medicago truncatula. Plant Mol Biol 54: 583–596

Lu M, Chai J, Fu D (2009) Structural basis for autoregulation of the zinc transporter YiiP. Nat Struct Mol Biol 16: 1063-1067

Lu Y (2010) Metal ions as matchmakers for proteins. Proc Natl Acad Sci USA 107:1811-1812.
Luk E, Jensen LT, Culotta VC (2003) The many highways for intracellular trafficking of metals. J Biol Chem 8: 803–809

Luo Z, Matthews AM, Weiss SR (1999) Amino acid substitutions within the leucine zipper domain of the murine coronavirus spike protein cause defects in oligomerization and the ability to induce cell-to-cell fusion. J Vir 73: 8152-8159.

MacDiarmid CW, Milanick MA, Eide DJ (2003) Induction of the *ZRC1* metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J Biol Chem 278: 15065–15072

Marschner H (1995) Mineral nutrition of higher plants. 2 ed. Academic Press, New York

Masuda H, Usuda K, Kobayashi T, Ishimaru Y, Kakei Y, Takahashi M, Higuchi K, Nakanishi H, Mori S, Nishizawa NK (2009) Overexpression of the barley nicotianamine synthase gene *HvNAS1* increases iron and zinc concentrations in rice grains. Rice 2: 155-166

Mäser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of Arabidopsis. Plant Physiol 126: 1646-1667

Mikkelsen MD, Pedas P, Schiller M, Vincze E, Mills RF, Borg S, Møller A, Schjoerring JK, Williams LE, Baekgaard L, Holm PB, Palmgren MG (2012) Barley HvHMA1 is a heavy metal pump involved in mobilizing organellar Zn and Cu and plays a role in metal loading into grains. Plos One **7**, e49027

Mills RF, Doherty ML, Lopez-Marques RL, Weimar T, Dupree P, Palmgren MG, Pittman JK, Williams LE (2008) ECA3, a Golgi-localized P2A-type ATPase, plays a crucial role in manganese nutrition in Arabidopsis. Plant Physiol 146: 116-128

Mills RF, Valdes B, Duke M, Peaston KA, Lahner B, Salt DE, Williams LE (2010) Functional significance of AtHMA4 C-terminal domain in planta. PLoS One 5, e13388

Mills RF, Peaston KA, Runions J, Williams LE (2012) HvHMA2, a P(1B)-ATPase from barley, is highly conserved among cereals and functions in Zn and Cd transport. PLoS One 7, e42640.

Milner MJ, Craft E, Yamaji N, Koyama E, Ma JF, Kochian LV (2012) Characterization of the high affinity Zn transporter from *Noccaea caerulescens*, NcZNT1, and dissection of its promoter for its role in Zn uptake and hyperaccumulation. New Phytol 195: 113-123

Montanini B, Blaudez D, Jeandroz S, Sanders D, Chalot M (2007) Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: improved signature and prediction of substrate specificity. BMC Genomics 8: 107

Moran R, Porath D (1980) Chlorophyll determination in intact tissues using n,n-dimethylformamide. Plant Physiol 65: 478-479

Murashige T, Skoog FA (1962) A revised medium for a rapid growth and bioassays with tobacco tissues cultures. Plant Physiol 15: 473-479

Nies DH, Silver S (1995) Ion efflux systems involved in bacterial metal resistances. J Ind Microbiol 14: 186-199.

Oomen RJ, Wu J, Lelièvre F, Blanchet S, Richaud P, Barbier-Brygoo H, Aarts MG, Thomine S (2009) Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thlaspi caerulescens*. New Phytol 181: 637-650

Outten CE, O'Halloran TV (2001) Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292: 2488–2492

Palmgren MG, Clemens S, Williams LE, Kramer U, Borg S, Schjorring JK, Sanders D (2008) Zinc biofortification of cereals: problems and solutions. Trends Plant Sci 13: 464-473

Pfeiffer WH, McClafferty B (2008) Biofortification: breeding micronutrient- dense crops. Breeding Major Food Staples: Blackwell Publishing Ltd. 61–91

Paulsen IT, Saier MH (1997) A novel family of ubiquitous heavy metal ion transport proteins. J Membr Biol 156, 99–103

Peiter E, Yan F, Schubert S (2000) J Plant Nutr 23:617–635

Peiter E, Montanini B, Gobert A, Pedas P, Husted S, Maathuis FJM, Blaudez D, Chalot M, Sanders D (2007) A secretory pathway-localized cation diffusion facilitator confers plant manganese tolerance. Plant Biol 104:8532–8537

Pittman JK, Shigaki T, Marshall JL, Morris JL, Cheng NH, Hirschi KD (2004) Functional and regulatory analysis of the Arabidopsis thaliana CAX2 cation transporter. Plant Mol Biol 56: 959–971

Podar D, Scherer J, Noordally Z, Herzyk P, Nies D, Sanders D (2012) Metal selectivity determinants in a family of transition metal transporters. J Biol Chem 287: 3185-3196

Prom-u-thai C, Huang L, Glahn RP, Welch RM, Fukai S, Rerkasem B (2006) Iron (Fe) bioavailability and the distribution of anti-Fe nutrition biochemicals in the unpolished, polished grain and bran fraction of five rice genotypes. J Sci Food Agr 86: 1209-1215

Quiquampoix H, Loughman BC, Rathcliffe RG (1993) J Exp Bot 44:1819–1827

Raboy V (2002) Progress in breeding low phytate crops. J Nutr 132: 503S-505S

Raboy V (2006) Seed phosphorus and the development of low-phytate crops. In: Turner BL, Richardson AE, Mullaney EJ (eds.) Inositol phosphates: linking agriculture and environment. CAB International, Wallingford, pp 111-132

Ramesh SA, Choimes S, Schachtman DP (2004) Over-expression of an Arabidopsis zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. Plant Mol Biol 54: 373-385

Ren M, Drivas G, D'Eustachio P, Rush MG (1993) Ran/TC4: a small nuclear GTP-binding protein that regulates DNA synthesis. J Cell Biol 120: 313-23

Ren Y, Liu Y, Chen H, Li G, Zhang X, Zhao J (2012) Type 4 metallothionein genes are involved in regulating Zn ion accumulation in late embryo and in controlling early seedling growth in Arabidopsis. Plant Cell Environ 35: 770-789

Rengel Z (2000) Manganese uptake and transport in plants. In: Sigel A, Sigel H, eds. Metal ions in biology systems, Vol. 37. New York, NY, USA: Marcel Dekker, 57–87

Schaaf G, Catoni E, Fitz M, Schwacke R, Schneider A, von Wirén N, Frommer WB (2002) A putative role for the vacuolar calcium/manganese proton antiporter AtCAX2 in heavy metal detoxification. Plant Biol 4: 612–618

Schaaf G, Ludewig U, Erenoglu BE, Mori S, Kitahara T, von Wiren N (2005) ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J Biol Chem 279: 9091–9096

Shahzad Z, Gosti F, Frerot H, Lacombe E, Roosens N, Saumitou-Laprade P, Berthomieu P (2010) The five AhMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in *Arabidopsis halleri*. PLoS Genetics **6**, e1000911.

Shingu Y, Kudo T, Ohsato S, Kimura M, Ono Y, Yamaguchi I, Hamamoto H (2005) Characterization of genes encoding metal tolerance proteins isolated from *Nicotiana glauca* and *Nicotiana tabacum*. Biochem Biophys Res Comm 331: 675– 680

Sinclair SA, Krämer U (2012) The zinc homeostasis network of land plants. Biochim Biophys Acta. doi: 10.1016/j.bbamcr.2012.05.016

Sperotto RA, Ricachenevsky FK, Duarte GL, Boff T, Lopes KL, Sperb ER, Fett JP (2009) Identification of up-regulated genes in flag leaves during rice grain filling and characterization of OsNAC5, a new ABA-dependent transcription factor. Planta 230: 985-1002

Sperotto RA, Ricachenevsky FK, Waldow Vde A, Fett JP (2012) Iron biofortification in rice: it's a long way to the top. Plant Sci 190: 24-39

Supek F, Supekova L, Nelson H, Nelson N (1996) A yeast manganese transporter related to the macrophage protein involved in conferringresistance to mycobacteria. Proc Natl Acad Sci USA 93: 5105–5110

Takahashi M, Terada Y, Nakai I, Nakanishi H, Yoshimura E, Mori S, Nishizawa NK (2003) The role of nicotianamine in the intracellular delivery of metals and plant reproductive development. Plant Cell 15: 1263-1280

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.

Tauris B, Borg S, Gregersen PL, Holm PB (2009) A roadmap for zinc trafficking in the developing barley grain based on laser capture microdissection and gene expression profiling. J Exp Bot 60: 1333-1347

Thomine S, Wang RC, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proc Natl Acad Sci USA 97: 4991–4996

Toenniessen GH (2002) Crop genetic improvement for enhanced human nutrition. J Nutr 132: 2943S-2946S

Tohme J, Jones P, Beebe S, Iwanaga M (1994) The combined use of agroecological and characterization data to establish the CIAT *Phaseolus vulgaris* core collection. In: Hodgkin T, Brown AHD, van Hintium TJL, Morales EAV (eds.) Core Collections of Plant Genetic Resources, J. Wiley & Sons, Chichester, UK. pp. 95-107

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J (2006) A *NAC* gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science 314: 1298-1301

van de Mortel JE, Almar Villanueva L, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MG (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. Plant Physiol 142: 1127-1147

van der Zaal BJ, Neuteboom LW, Pinas JE, Chardonnens AN, Schat H, Verkleij JA, Hooykaas PJ (1999) Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Phys 119: 1047-1055.

Vasconcelos M, Datta K, Oliva N, Khalekuzzaman M, Torrizo L, Krishnan S, Olivera M, Goto F, De Datta SK (2003) Enhanced iron and zinc accumulation in transgenic rice with the ferritin gene. Plant Science 164: 371-378

Vert G, Grotz N, Dédaldéchamp F, Gaymard F, Guerinot ML, Briat JF, Curie C (2002) IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth. Plant Cell 14: 1223-1233

Verret F, Gravot A, Auroy P, Preveral S, Forestier C, Vavasseur A, Richaud P (2005) Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His11 stretch. FEBS Lett 579: 1515-1522

Wang F, Dufner-Beattie J, Kim BE, Petris MJ, Andrews G, Eide DJ (2004) Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J Biol Chem 279: 24631–24639

Waters BM, Grusak MA (2008) Quantitative trait locus mapping for seed mineral concentrations in two *Arabidopsis thaliana* recombinant inbred populations. New Phytol 179: 1033-1047

Weber M, Harada E, Vess C, Roepenack-Lahaye E, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J 37: 269-281

Wei Y, Fu D (2005) Selective metal binding to a membrane-embedded aspartate in the *Escherichia coli* metal transporter YiiP (FieF). J Biol Chem 280: 33716–33724

Welch RM (2002) Breeding strategies for biofortified staple plant foods to reduce micronutrient malnutrition globally. J Nutr 132: 495S-499S

Welch RM, Graham RD (2004) Breeding for micronutrients in staple food crops from a human nutrition perspective. J Exp Bot 55: 353-364.

Williams LE, Mills RF (2005) P(1B)-ATPases--an ancient family of transition metal pumps with diverse functions in plants. Trends Plant Sci 10: 491-502

Wirth J, Poletti S, Aeschlimann B, Yakandawala N, Drosse B, Osorio S, Tohge T, Fernie AR, Gunther D, Gruissem W, Sautter C (2009) Rice endosperm iron biofortification by targeted and synergistic action of nicotianamine synthase and ferritin. Plant Biotech J 7: 631-644

Wong CKE, Cobbett CS (2009) HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in Arabidopsis thaliana. New Phytol 181: 71-78

Wu ZY, Liang F, Hong BM, Young JC, Sussman MR, Harper JF, Sze H (2002) An endoplasmic reticulum-bound Ca2+/Mn2+ pump, ECA1, supports plant growth and confers tolerance to Mn2+ stress. Plant Phys 130: 128–137

Yang X, Huang J, Jiang Y, Zhang HS (2009) Cloning and functional identification of two members of the ZIP (Zrt, Irt-like protein) gene family in rice (Oryza sativa L.). Mol Biol Rep 36: 281-287

Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, et al (2002) A draft sequence of the rice genome (Oryza sativa L. ssp. indica). Science 296: 79-92

Yuan L, Yang S, Liu B, Zhang M, Wu K (2012) Molecular characterization of a rice metal tolerance protein, OsMTP1. Plant Cell Rep 31: 67-79

Zhang Y, Xu YH, Yi HY, Gong JM (2012) Vacuolar membrane transporters OsVIT1 and OsVIT2 modulate iron translocation between flag leaves and seeds in rice. Plant J. doi: 10.1111/j.1365-313X.2012.05088.x

Zhao H, Eide D (1996a) The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc Natl Acad Sci USA 93: 2454–2458

Zhao H, Eide D (1996b) The *ZRT2* gene encodes the low affinity zinc transporter in Saccharomyces cerevisiae. J Biol Chem 271: 23203–23210