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Bruno Bachiega Navarro

REGULAÇÃO DA HOMEOSTASE DE COBRE EM PLANTAS DE ARROZ

Santa Maria, RS
2020

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Dissertação de Mestrado apresentado ao Programa de Pós-Graduação em Agrobiologia da Universidade Federal de Santa Maria (UFSM), como requisito para a obtenção parcial do título de **Mestre em Agrobiologia**.

Orientador: Dr. Felipe Klein Ricachenevsky
Coorientador: Dr. Raul Antonio Sperotto

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Santa Maria, RS
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RESUMO

REGULAÇÃO DA HOMEOSTASE DE COBRE EM PLANTAS DE ARROZ

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O arroz é o segundo cereal de maior importância para a alimentação humana, contribuindo com 19% de calorias consumidas por humanos. Porém, o cultivo do arroz pode ser afetado por diferentes variações ambientais, dentre elas a variação na disponibilidade de nutrientes. O cobre (Cu) é essencial para a fotossíntese, além de atuar como cofator de diversas enzimas. A deficiência de Cu desencadeia uma cascata de sinalização para aquisição de Cu, visando manter a eficiência fotossintética da planta. O objetivo deste estudo foi verificar os possíveis genes envolvidos no mecanismo de economia de Cu em plantas de arroz, utilizando para isso o mutante para a proteína OsHMA5 (*oshma5*) e seu respectivo tipo selvagem cv. Nipponbare (WT) submetidos a deficiência de Cu. Plantas foram submetidas a condição controle (CC) e deficiência de cobre (-Cu) por 15 dias. Foram mensuradas as raízes e folhas, nas quais foram realizadas quantificação de elementos e extração de RNA para síntese de cDNA e análise de expressão gênica. Os resultados mostram que *oshma5* apresentou menor crescimento da parte aérea em comparação ao tipo selvagem tanto na condição controle quanto em deficiência. Foram observadas alterações na partição de elementos de acordo com a condição e genótipos, em especial um maior acúmulo de Fe na parte aérea de *oshma5* sob –Cu, além de redução de Cu na parte aérea em *oshma5* sob –Cu.Também observamos maior acumulo de Mn na parte aérea de *oshma5* em ambas as condições, além disso uma redução de Cu e aumento de Fe nas sementes de *oshma5*. Com relação a expressão gênica, plantas em condição de deficiência apresentaram maior expressão de COPT1 e COPT5 nas folhas e COPT5 nas raízes. Plantas *oshma5* tiveram uma menor expressão dos genes CSD1, CSD2, CSD3 em folhas e uma maior expressão nas raízes em –Cu com relação ao WT. Em contrapartida, FSD1 e FSD2 nas folhas e FSD2 nas raízes tiveram uma maior expressão no *oshma5* comparado ao WT. Os miRNAs 397ab, 398ab e 408 tiveram um aumento expressivo em condição de deficiência em ambos os genótipos. A expressão de genes relacionados a absorção e armazenamento de Fe foram expressos de forma diferenciada de acordo com a condição, órgão e genótipo. Esses dados mostram pela primeira vez indícios de que o arroz pode ser um mecanismo de economia de cobre semelhante ao relatado em *A. thaliana*.

Palavras-chave: Deficiência de cobre, economia de cobre, oshma5, *Oryza sativa*

ABSTRACT

COPPER REGULATION HOMEOSTASIS IN RICE PLANTS

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Rice is the second most important cereal for human intake, responsible for 19% of calories consumed by humans. However, rice growth may be affected by environmental variations, among them the availability of nutrients. Copper (Cu) is essential for photosynthesis, as well as a cofactor for several enzymes. Copper deficiency triggers a cascade of signaling for Cu acquisition to maintain the photosynthetic efficiency of the plant. The goal of this study was to verify the possible genes involved in the mechanism of Cu deficiency in rice plants, using the mutant for the protein OsHMA5 (*oshma5*) and its respective wild type cv. Nipponbare (WT) submitted to Cu deficiency. Plants were submitted to control condition (CC) and copper deficiency (-Cu) for 15 days. The roots and leaves were measured, in which element quantification and RNA extraction were performed for cDNA synthesis and gene expression analysis. The results showed that *oshma5* had lower shoot growth compared to the wild type in both control and deficiency conditions. Changes in element partition according to condition and genotypes were observed, especially a greater accumulation of Fe in shoots of oshma5 under –Cu. Reduction of Cu in shoot of oshma5 under –Cu and greater accumulation of Mn in shoots of *oshma5* under both conditions. In addition, *oshma5* reduction of Cu and an increase of Fe in seeds. Regarding gene expression, plants in deficiency condition had a higher expression of COPT1 and COPT5 in the leaves and COPT5 in the roots. *Oshma5* had a lower expression of CSD1, CSD2, CSD3 genes in shoots and a higher expression in roots under –Cu in comparison to WT. In contrast, FSD1 and FSD2 on shoots and FSD2 on roots had a higher expression in *oshma5* compared to WT. The miRNAs 397ab, 398b and 408 had a significant increase in deficiency condition in both genotypes. The expression of genes related to absorption and storage of Fe were expressed differently according to condition, organ and genotype. These data show for the first time evidences that rice may be have a similar mechanism of copper economy to that reported in *A. thaliana*.

Keywords: Copper deffiency, Copper economy, *oshma5*, *Oryza sativa*

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LISTA DE ABREVIATURAS E SIGLAS

AHA	*Arabidopsis proton ATPase*
At	*Arabidopsis thaliana*
ATP	Adenosina trifosfato *(Adenosine triphosphate)*
CCS	Chaperona de cobre (*Copper chaperone)*
cDNA	DNA complementar (*Complementary DNA*)
COPT/CTR	Proteínas transportadoras de Cu (*COPper Transporter protein)*
CRR	Regulador de resposta de Cu (*Cu response regulator)*
CSD	Cobre-zinco Superóxido dismutase (copper-*Zinc Superoxide dismutase)*
Cu	Cobre (*Copper)*
CuRE	Elementos responsivos ao cobre (*Copper responsive elements*)
cv.	Cultivar
CYC6	Citocromo *c* 6
DNA	Ácido desoxirribonucleico *(Deoxyribonucleic acid)*
EROs	Espécies reativas de oxigênio (Reactive oxygen species)
ETR	Responsivo ao etileno (*Ethylene responsive)*
Fe	Ferro (*Iron)*
FeSOD (FSD)	Ferro superóxido dismutase (*Iron superoxide dismutase*)
FRO	Oxidase-redutase férrica (*Ferric redutase oxidase)*
HMA	Domínio associado a metal pesado (*Heavy Metal Associated domain*)
IRO	Fator de transcrição relacionada a proteinas de ferro (*Protein IRON-RELATED TRANSCRIPTION FACTOR*)
IRT	Transportar regulado por ferro *(Iron-regulated transporter)*
miRNA	MicroRNA
Mn	Manganês (*Manganese)*
MnSOD	Manganês superóxido dismutase (Manganese superoxide dismutase)
mRNA	RNA mensangeiro *(Messenger RNA)*
MS	Matéria seca *(Dry weight)*
NRAMP	Proteína de macrófago associada à resistência natural (natural resistance-associated macrophage protein)
Os	*Oryza sativa*
PCR	Reação em cadeia da polimerase (*Polymerase chain reaction*)
PPO	Polifenol oxidase (*polyphenol oxidase)*
PSII	Fotossistema II (*Photosystem II*)
RAN	Responsivo ao antagonista *(Responsive to antagonist)*
RNA	Ácido ribonucleico *(Ribonucleic acid)*
RT-qPCR	PCR em tempo real quantitativo com transcrição reversa *(Real Time Quantitative Reverse Transcription PCR)*
SBP	Promotor Squamosa ligante a proteínas (*Squamosa promoter binding proteins)*

SOD	Superóxido dismutase *(Superoxide dismutase)*
SPL	Promotor semelhante à Squamosa ligante a proteínas (*Squamosa promoter binding like protein)*
Tos17	Transposon de *Oryza sativa* 17
VIT	Transportador vacuolar de ferro *(Vacuolar Iron transporter)*
WT	Tipo selvagem *(Wild type)*
YSL	Proteína semelhante à yellow stripe (*yellow stripe-like protein*)
ZIP	Proteína ZRT-IRT (*zinc-regulated transporters- iron-regulated transporter*)
Zn	Zinco / *Zinc*

SUMÁRIO

INTRODUÇÃO

1.1 ARROZ

O arroz (*Oryza sativa*) é o segundo cereal mais importante do mundo para o consumo humano. É considerado o alimento base para mais da metade população mundial, contribuindo com 80 % do consumo de calorias nos países Asiáticos e mais de 23% da população mundial (PANDEY et al., 2010). Entretanto, possui baixas concentrações de nutrientes como Fe e Zn (JOHNSON et al., 2011; MORENO-MOYANO et al., 2016).

Na América, o Brasil é principal produtor de arroz sendo cultivado em diferentes ecossistemas, desde a região tropical com arroz de sequeiro, como a região temperada com arroz cultivado em terrenos alagado (GADAL et al., 2019). O gênero *Oryza* compreende 2 genótipos cultivados e mais 22 genótipos selvagens, representando diferentes grupos de genomas (AA, BB, CC, EE, FF, GG, BBCC, CCDD, KKLL e HHJJ) (SANCHEZ; WING; BRAR, 2013). A domesticação da *Oryza sativa* se deu a mais de 9 mil anos (DOGARA; JUMARE, 2014). *O. sativa* spp. *japonica* teve como centro de origem da sua domesticação no sul da China, enquanto que *O. sativa* spp. *indica* ocorreu no sul da Ásia (HUANG et al., 2012).

O. sativa tem o genoma diploide (2n=24) e apresenta o menor genoma entre os principais cereais (430 Mb) e contém aproximadamente 40.000 genes codificantes. Desta forma o arroz é considerado a planta modelo para estudos moleculares em *Poaceae* (GOFF et al., 2002).

1.2 COBRE

O cobre (Cu) é um dos microelementos considerado essencial para as plantas, atuando como cofator de diversas moléculas consideradas fundamentais para que a planta complete seu ciclo de vida (MARSCHNER, 2012). Entretanto, o Cu também pode ser um agente tóxico para as plantas quando em concentrações elevadas. Desta forma, as plantas desenvolveram mecanismos a fim de manter uma concentração adequada nas células e ao mesmo tempo minimizar a toxidez ou a deficiência de Cu (BURKHEAD et al., 2009). Além disso, devido à capacidade de alterar seu estado de oxidação entre as formas oxidada (Cu^{2+} - insolúvel) e reduzida (Cu^{+} - solúvel), o Cu exerce papel fundamental nas reações de transferência de elétrons (YRUELA, 2013). Na forma oxidada, o Cu possui maior afinidade com o nitrogênio do aminoácido histidina; já na forma reduzida, possui maior afinidade com o enxofre da cisteína e da metionina (HOLM; KENNEPOHL; SOLOMON, 1996). Assim, o Cu em condições adequadas, pode ser encontrado nos tecidos vegetais (98% do total) na forma complexada a proteínas e peptídeos (MEHARG, 2012; YRUELA, 2013). Abaixo descrevemos brevemente algumas das proteínas cúpricas conhecidas.

1.2.1 PLASTOCIANINAS

É a proteína cúprica mais abundante nas plantas, sendo composta por 97-104 aminoácidos; em plantas superiores, sua localização é em órgão verdes, associada a membrana dos tilacóides, nos cloroplastos. Sua função é vital para as plantas pois é responsável pelo transporte de elétrons do citocromo *f* do complexo *b6f* para o complexo $P700^{+}$ do fotossistema I (KATOH, 1977; REDINBO; YEATES; MERCHANT, 1994). Já as plantacianinas, similares à plastocianina são proteínas localizada no apoplasto do pólen, responsáveis por guiar o tubo polínico no estigma, e também requerem Cu para seu funcionamento adequado (DONG; KIM; LORD, 2005).

1.2.2 SUPERÓXIDO DISMUTASE (SOD)

São famílias de enzimas responsáveis pela dismutação do ânion superóxido (O_2^{-*}) ($2O_2^{-*} + 2H^+ \rightarrow H_2O_2 + O_2$) (FRIDOVICH, 1975). Já são descritos grupos de proteínas SOD que se distinguem pelo metal utilizado como grupo prostético: Cobre/Zinco –SOD (Cu/Zn-SOD), Ferro – SOD (FeSOD), Manganês – SOD (MnSOD) e Niquel – SOD (NiSOD), sendo este último ainda não encontrado em plantas (DUPONT et al., 2008; FINK; SCANDALIOS, 2002). Em *Arabidopsis thaliana* são encontradas três proteínas do subgrupo das Cu/Zn-SOD: AtCSD1, AtCSD2 e AtCSD3, localizadas no citoplasma, cloroplasto e peroxissomo, respectivamente (GILL et al., 2015). Em *Oryza sativa* já são conhecidas quatro proteínas CSD, com localizações sugeridas baseadas em predições computacionais: OsCSD1 no citosol, OsCSD2 no cloroplasto, OsCSD3 no peroxissomo e OsCSD4 também no citosol (KAMINAKA et al., 1997; KANEMATSU; ASADA, 1989; PAN; HWANG; LIU, 1999; PRAKASH SANYAL et al., 2018). No entanto, ainda se sabe pouco sobre as funções específicas destas enzimas em cada compartimento subcelular.

1.2.3 ASCORBATO OXIDASE (AO)

É uma glicoproteína que contém três domínios. É responsável pela oxidação do ácido ascórbico em ácido desidroascórbico. Esta enzima contém oito átomos de Cu por molécula. É encontrado em caules, flores, frutos e sementes em todas as fases do desenvolvimento e em células diferenciadas e indiferenciadas (MONDOVÌ; AVIGLIANO, 2018). No nível celular, foi localizada na parede celular e no citoplasma (MONDOVÌ; AVIGLIANO, 2018). A AO pode agir como oxidase terminal da cadeia respiratória ou em combinação com a enzima polifenol oxidase. A expressão de AO é induzida pela luz e pelo aparecimento de lesões. A enzima atua nos mecanismos de defesa contra os oxidantes relacionados ao ascorbato ou vitamina C (STEVENS et al., 2017).

1.2.4 CITOCROMO *C* OXIDASE (COX)

É uma oxidase terminal do transporte de elétrons mitocondrial. A energia produzida após este processo é utilizada para bombear prótons para a membrana mitocondrial externa e sintetizar ATP. Esta enzima possui em sua estrutura dois átomos Cu e dois átomos de Fe (heme). Quando esta enzima é inibida, há outra oxidase chamada "oxidase alternativa" que funciona como uma rota alternativa de oxidação na mitocôndria. Desta forma, não há bombeamento de prótons para o exterior. Portanto toda a energia produzida é perdida na forma de calor. Esta oxidase alternativa contém Cu mas não Fe em sua estrutura (WIKSTRÖM; KRAB; SHARMA, 2018).

1.2.5 LACASES

As lacases são uma família de glicoproteínas cúpricas com atividade oxidase. Essas enzimas, diferente da peroxidases, utilizam o oxigênio ao invés do peróxido na oxidação de substratos como compostos fenólicos e compostos inorgânicos (NAKAMURA; GO, 2005). Diversas lacases já foram relacionadas com a biossíntese de lignina e também em respostas ao ataque de patógenos e estresses abióticos (CAI et al., 2006; LIU et al., 2015, 2017). Em *A. thaliana* já foram descritos 17 genes para lacases (TURLAPATI et al., 2011). Dentre eles, os genes AtLAC2, AtLAC3, AtLAC4, AtLAC5, AtLAC12, AtLAC13 e AtLAC17 são modulados pela concentração de Cu, tendo sua expressão reduzida em deficiência de Cu (ABDEL-GHANY; PILON, 2008). Já mais recentemente, foi mostrado que em *O. sativa* há 30 genes para lacases (LIU et al., 2017). Dentre eles, OsLAC4, OsLAC5, OsLAC11, OsLAC12, OsLAC13, OsLAC30 tiveram sua expressão aumentada em deficiência de Cu, enquanto OsLAC3, OsLAC10, OsLAC23, OsLAC28 e OsLAC29 tiveram suas expressões aumentadas conforme o aumento de Cu no meio (LIU et al., 2017). Dentre as lacases, AtLAC2, AtLAC4 e AtLAC17 são moduladas por miRNA397 (BERTHET et al., 2011) e AtLAC3, AtLAC12 e AtLAC13 são moduladas por miRNA408 (ABDEL-GHANY; PILON, 2008).

1.3 DISPONIBILIDADE DO COBRE

A biodisponibilidade de micronutrientes localizados na rizosfera pode ser limitada devido à sua baixa solubilidade em solos aerados e também pela geração de uma forte ligação às partículas do solo formando assim um complexo estável (MCMANUS et al., 2018). Com isso, as plantas desenvolveram uma série de estratégias que possibilitam a absorção de micronutrientes. Dentre estes mecanismos, a acidificação da rizosfera promove a solubilização de alguns micronutrientes. Em *Arabidopsis thaliana*, membros da família H+-ATPase AHA (de Arabidopsis H+-ATPase) podem estar envolvidos na acidificação (FALHOF et al., 2016). H+-ATPases são bombas de prótons que estão localizadas na membrana e geram um potencial de membrana, bombeando prótons para o meio externo, favorecendo o transporte de solutos carregados positivamente para dentro da célula (FALHOF et al., 2016). Outra forma é a redução do metal; a família de metal redutase FRO (*ferric redutase oxidase*) foi identificada em *A. thaliana*, e é composta por 8 membros (MUKHERJEE et al., 2006). A proteína AtFRO2 é responsável pela redução de Fe^{3+} a Fe^{2+}, e tem expressão aumentada sob deficiência de Fe (KOBAYASHI; NAKANISHI; NISHIZAWA, 2010). Com respeito ao AtFRO3, sua expressão é aumentada em raízes de *A. thaliana* quando em deficiência de Fe e Cu, sugerindo um papel para a AtFRO3 na aquisição tanto do Fe como Cu (JAIN; WILSON; CONNOLLY, 2014).

Interessantemente, a expressão dos genes FRO em diferentes órgãos sugere que há necessidade da atividade de redutase não só nas raízes (MUKHEmRJEE et al., 2006). Há evidências de que a deficiência de Cu induz um aumento na atividade de redutase na membrana mais externa da raiz paralelamente com a acidificação da rizosfera. Ambos as respostas são independentes e poderiam contribuir separadamente para a absorção de íons metálicos (YI; GUERINOT, 1996). Em contrapartida, foi verificado que em mutantes de *A. thaliana frd1* a redução do Cu na rizosfera não é essencial para a absorção do Cu (YI; GUERINOT, 1996). Mutantes individuais de *A. thaliana fro4* e *fro5* tiveram uma drástica redução da absorção de Cu quando comparado ao selvagem, e quando foi gerado duplo mutante *fro4/fro5* a absorção de Cu foi extremamente baixa, chegando a níveis quase não detectáveis, sugerindo que os genes AtFRO4 e AtFRO5 possuem um papel importante na redução do Cu na rizosfera e consequentemente na absorção de Cu^{+1} (BERNAL et al., 2012).

1.4 DEFICIÊNCIA DE COBRE

Os sintomas da deficiência de Cu são inicialmente manifestados nas folhas jovens. O fenótipo de deficiência é caracterizado pelo surgimento de manchas cloróticas nas pontas das folhas que mais tarde se estendem até as margens. As folhas tendem a enrolar e apresentar um aspecto deformado, flácido e eventualmente necrótico (MARSCHNER, 2012). Em condições de deficiência extrema, ocorre abscisão prematura das folhas, assim como aborto do meristema apical e radicular. Nos cereais, as plantas em fase reprodutiva são mais sensíveis a deficiência de Cu do que plantas em estágio vegetativo. Os sintomas mais comuns das plantas cultivadas em condições de deficiência são murchidão, atraso da maturidade, esterilidade masculina, redução do peso e tamanho dos grãos, o que pode ocasionar perdas importantes na produção de sementes (GRAHAM, 1975; PANDEY, 2018). Este fenômeno deve-se ao fato de o Cu ser necessário para a lignificação da antera. Entretanto, a deficiência de Cu pode ser corrigida através da aplicação de fertilizantes no solo ou pulverizando diretamente nas folhas (PANDEY, 2018).

Em geral, sabe-se que existe mecanismos moleculares em resposta a deficiência de Cu. Estes mecanismos incluem mudanças na expressão gênica e na morfologia das plantas para melhorar a aquisição, e realocar o uso do Cu de funções menos essenciais para as mais essenciais. Esta alterações são chamadas de “Mecanismo de Economia de Cobre” (PRINTZ et al., 2016; SOMMER et al., 2010).

Em *Chlamydomonas*, sob condições de deficiência de Cu, a substituição das proteínas contendo Cu por proteínas contendo Fe funcionalmente equivalentes está bem documentada (SOMMER et al., 2010). Por exemplo, as células substituem a plastocianina dentro da cadeia fotossintética pelo CYC6 (citocromo *c* 6), uma proteína heme independente de Cu (KROPAT et al., 2005). As plantas superiores não substituem a plastocianina quando em deficiência de Cu, pois é essencial para estes organismos (WEIGEL et al., 2003), mas a Cu/ZnSOD (CSD2) é substituída por FeSOD (FSD1, Fe superoxide dismutase) em *A. thaliana* (ABDEL-GHANY et al., 2005a). Esta regulação coordenada provavelmente permite melhor uso dos metais disponíveis no cloroplasto, sugerindo que os níveis de Cu no estroma regulam a expressão nuclear destes genes (BURKHEAD et al., 2009; YAMASAKI; PILON; SHIKANAI, 2008).

O gene CRR1 (*copper response regulator 1*) foi identificado como um dos responsáveis pelo processo de ativação gênica induzida por deficiência de Cu em *Chlamydomonas* (ERIKSSON et al., 2004; SOMMER et al., 2010). A proteína *Crr1* possui um domínio de ligação

de DNA chamado SBP (*Squamosa binding protein*) (ERIKSSON et al., 2004; SOMMER et al., 2010). Os ensaios mostraram que em deficiência de Cu, o domínio SBP e *Crr1* se liga especificamente aos elementos chamados de CuREs (*Copper responsive elements*) os quais apresentam um domínio de ligação GTAC dentro da região promotora, induzindo a transcrição de CYC6 (KROPAT et al., 2005). O genoma de *A. thaliana* contém 17 proteínas com domínio SPL (*Squamosa protein like*), e o de arroz, 19 (BIRKENBIHL et al., 2005; YANG et al., 2008).

Em *A. thaliana*, a resposta a deficiência de Cu parece estar ligada à regulação pós-transcricional por miRNAs (BURKHEAD et al., 2009). Em geral, o mecanismo de economia de cobre envolve três passos; o primeiro é através da ativação de proteínas de aquisição de Cu de alta afinidade nas raízes, por meio de membros da família COPT/Ctr; o segundo envolve um aumento da expressão de miRNAs que vão se ligar a mRNA de proteínas cúpricas menos essenciais; terceiro, a substituição envolvendo enzimas de defesa, reduzindo a expressão de Cu/Zn-SOD e aumentando a de Fe-SOD (ABDEL-GHANY; PILON, 2008; BURKHEAD et al., 2009; PILON, 2017; PRINTZ et al., 2016). Entretanto, pouco se sabe se este mecanismo é conservado em outras plantas. **No table of figures entries found.**

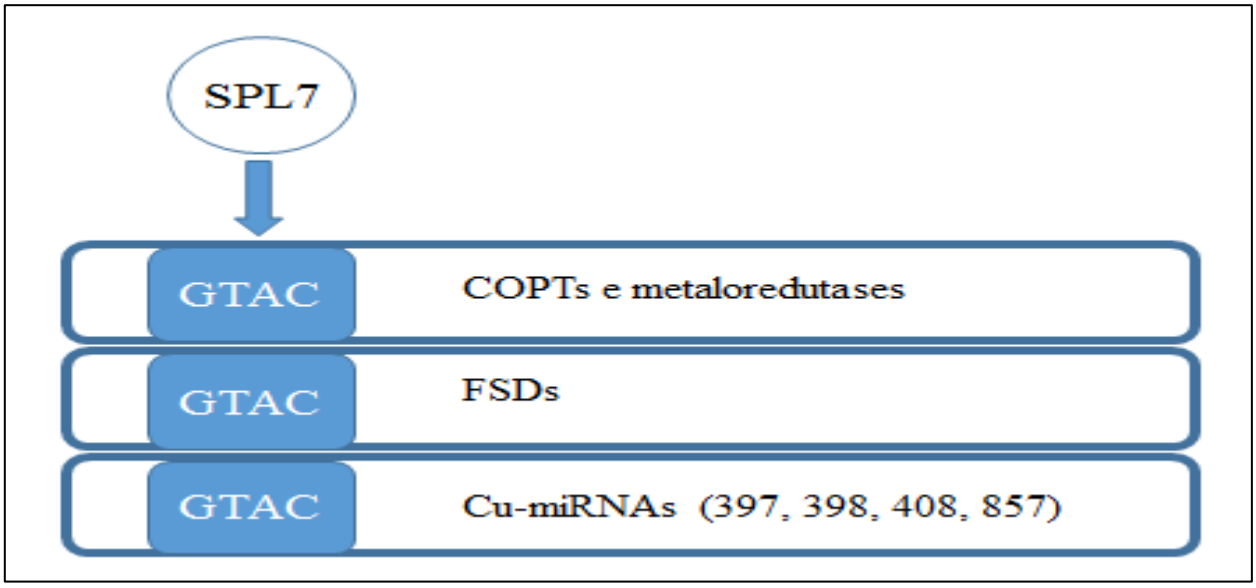

Figura 1. Esquema do mecanismo de economia de cobre em *A. thaliana*: 1) O SPL7 se liga ao motivo GTAC nos elementos de resposta ao cobre no promotor de genes responsivos ao cobre e ativa a expressão de genes de alta afinidade de cobre como COPT1 e COPT5 e de metaloredutases; 2) Aumenta da expressão de genes (FSDs e MnSD) que utilizam outro metal como cofator mas que possuem funções iguais a CuSOD; 3) Aumento da expressão de miRNA

que tem como alvos mRNAs de proteinas cúpricas não essenciais, assim realocando cobre para próteinas essenciais como Plastocianina. Adaptado de (ARAKI et al., 2018; BERNAL et al., 2012; PRINTZ et al., 2016; YAMASAKI et al., 2009).

1.5 FAMÍLIAS GÊNICAS ENVOLVIDAS NA HOMEOSTASE DE COBRE

1.5.1 P_{1B} – ATPASES

Os transportadores da família P_{1B}-ATPase, também conhecidos como HMAs ("*heavy metal ATPases*") utilizam a energia da hidrólise da molécula de ATP para bombear cátions através da membrana (BAXTER et al., 2003). Estruturalmente, os P_{1B}-ATPases possuem 8 hélices transmembranares e têm características comuns, tais como o domínio de ligação ATP (GDxxNDxP) ou o domínio da fosforilação (DKTGT). Em particular, as proteínas do grupo das ATPases do tipo P_{1B} apresentam especificidade por metais de transição, sendo divididos em dois subgrupos de acordo com a valência dos metais (monovalente e bivalente) (BAXTER et al., 2003). O genoma de *A. thaliana* codifica para 8 proteínas HMA (AtHMA1-8), enquanto o genoma de *O. sativa* codifica 9 proteínas (OsHMA1-9) (AXELSEN; PALMGREN, 1998; BAXTER et al., 2003). O primeiro gene desta família funcionalmente caracterizado em *A. thaliana* foi o HMA7 ou também chamado de RNA1 ("*responsive-to-antagonist* 1") (HIRAYAMA et al., 1999). O HMA7 (RAN1) tem como função transportar Cu para o receptor de etileno localizado no retículo endoplasmático (ETR1 – *Ethylene responsive* 1) (CHEN et al., 2002; HIRAYAMA et al., 1999).

AtHMA1 é membro da família P1B-ATPase, localizado no envelope do cloroplasto, e está envolvido no influxo de Cu para o estroma (SEIGNEURIN-BERNY et al., 2006). Mutantes neste transportador apresentam diminuição da atividade do Cu/Zn-SOD no cloroplasto, sugerindo que o AtHMA1 libera Cu para Cu/Zn-SOD preferencialmente. AtHMA1 não contém a região típica de ligação à Cu^{+}, MxCxxM, na sua extremidade N-terminal, porém é rica em histidina, o que sugere uma preferência pelo transporte de Cu^{2+} em vez de Cu^{+} (SEIGNEURIN-BERNY et al., 2006). AtHMA1 também atua no bombeamento de Ca^{2+} na célula (MORENO et

al., 2008), e no efluxo de Zn^{+2} no cloroplasto, sugerindo sua importância na detoxificação de Zn no estroma (KIM et al., 2009).

O transporte de Cu no cloroplasto depende das proteínas P1B-ATPase PAA1 ("*P-type ATPase for Arabidopsis*") ou AtHMA6, e PAA2 ou AtHMA8. AtHMA8 se localiza na membrana do tilacoide, enquanto que AtHMA6 se localiza na membrana do cloroplasto (MAYERHOFER et al., 2016; SAUTRON et al., 2015). Ambas as ATPases são necessárias para liberar Cu para o tilacóide, enquanto apenas o mutante HMA6 mostra uma diminuição na liberação de Cu para a enzima Cu/Zn-SOD do estroma (ABDEL-GHANY et al., 2005b). Em *A. thaliana*, AtHMA6 é expresso tanto em raízes como em parte aérea, enquanto AtHMA8 só é detectado na parte aérea (ABDEL-GHANY et al., 2005b). Em *O. sativa* o gene OsHMA6 está localizado na membrana plasmática (WENLI et al., 2020). Analisando a topologia da proteína OsHMA6, foi observado uma semelhança de 82,78% na sequência de aminoácidos com a proteína OsHMA9, sugerindo que as duas proteínas possam apresentar funções semelhantes de efluxo de Cu na célula; entretanto, OsHMA6 é expresso em todos órgãos em diferentes estádios de desenvolvimento, já o OsHMA9 tem expressão localizada nas raízes, nas bainhas foliares e no limbo foliar (LEE et al., 2007; WENLI et al., 2020). A expressão desses genes é modulado de acordo com a concentração de Cu no meio (WENLI et al., 2020).

Em *A. thaliana*, o transportador AtHMA5 tem expressão principalmente nas raízes e flores (ANDRÉS-COLÁS et al., 2006; KOBAYASHI et al., 2008). Foi mostrado que a função de AtHMA5 é regulada de acordo com os níveis de Cu: em baixas concentrações estaria mais expresso no complexo de Golgi, enquanto que em altas concentrações ele funciona na detoxificação do Cu nas células. Por meio da técnica duplo-híbrido foi possível descrever que o AtHMA5 interage com duas chaperonas: ATX1 e CCS no domínio C-terminal específico da proteína. Por outro lado, o AtHMA5 também pode desempenhar um papel na expansão celular (ANDRÉS-COLÁS et al., 2006). Em *O. sativa* o gene OsHMA5 foi caracterizado em detalhe. OsHMA5 é expresso nas células do periciclo durante o estádio vegetativo, enquanto no estádio reprodutivo foi observado também expressão nos nós e pedúnculo (DENG et al., 2013). Mutações no gene OsHMA5 diminuem a concentração de Cu na parte aérea, sugerindo que esta proteína tem função no carregamento de Cu no xilema, translocando Cu para a parte aérea, sendo ótimo modelo para estudos da homeostase de Cu (DENG et al., 2013).

1.5.2 COPTS

Os transportadores COPT (*Copper transporter family*) são homólogos das proteínas transportadoras CTR encontradas em leveduras. Estruturalmente, elas possuem três hélices transmembrana contendo na sua extremidade N-ternimal uma sequência rica em metionina, a qual é facilmente atraída por íons de Cu e provavelmente facilita seu transporte (PUIG; THIELE, 2002). Seis membros desta família foram identificados em *A. thaliana* (AtCOPT1-6), enquanto que em arroz já foram identificados 7 membros (OsCOPT1-7). Eles são responsáveis pelo transporte do Cu através das membranas plasmática de diferentes tecidos e compartimentos sub-celulares (SANCENÓN et al., 2004; YUAN et al., 2011).

Especificamente, AtCOPT1 é um transportador de Cu de alta afinidade que está localizado na membrana plasmática de células radiculares, células-guarda, tricomas, pólen e embriões, tendo como função a aquisição de Cu do solo, o desenvolvimento do pólen e alongamento das raízes (SANCENÓN et al., 2004). O gene AtCOPT1 tem sua expressão aumentada quando em deficiência Cu (PEÑARRUBIA et al., 2010). Em *A. thaliana* foi demonstrado que AtCOPT2 está localizado na membrana plasmática e tem como função a aquisição de Cu e uma possível relação com respostas de deficiência de Fe (PEREA-GARCÍA et al., 2013). AtCOPT6 está localizado na membrana plasmática e é expresso tanto em deficiência quando em excesso de Cu (GARCIA-MOLINA et al., 2013). Já AtCOPT3 está localizado na membrana do cloroplasto, e COPT5 está localizado na membrana do vacúolo, sendo responsável pelo efluxo de Cu do tonoplasto, possivelmente envolvido na translocação de Cu para os órgãos reprodutivos (CARRIÓ-SEGUÍ et al., 2019; KLAUMANN et al., 2011).

1.5.3 miRNAS

Os MicroRNAs (miRNAs) são pequenos RNAs que estão envolvidos na regulação pós transcripcional da expressão gênica. Atualmente, de acordo a miRBase *website* são anotados 428 miRNAs maduros para *A. thaliana* e 738 em *O. sativa*. A biogênese do MiRNA se inicia com a transcrição dos genes MIR pela RNA polimerase II em transcritos longos de RNA primário (pri-miRNA), que se dobram em estruturas de grampos (*hairpin*). Os pri-miRNAs são clivados por enzimas do tipo RNAse III, tipicamente do tipo DICER 1 (DCL1 – *Dicer-like 1*) em um processo

de duas etapas para produzir estruturas menores chamado de miRNAs precursores (pre-miRNAs), que são subsequentemente processados para produzir um duplex de miRNA (miRNA 5'/miRNA 3') (BUDAK; AKPINAR, 2015; ROGERS; CHEN, 2013). Embora o DCL1 seja a principal proteína tipo DICER envolvida na biogênese do miRNA, DCL3 e DCL4 também são capazes de processar transcritos de precursores de miRNA (BUDAK; AKPINAR, 2015; CUPERUS; FAHLGREN; CARRINGTON, 2011). O dúplex é metilado em ambas as fitas pelo *Hua enhancer 1* (HEN1) na sua extremidade de 3' para proteger os miRNAs da degradação de 3'-exonucleases. O dúplex é então transferido para o citoplasma por uma proteína de membrana nuclear conhecida como *HASTY* (HST), onde a fita do miRNA maduro liga a proteína Argonauta (AGO1) no complexo induzido pelo RNA (RISC) (BUDAK; AKPINAR, 2015). AGO1 é o principal Argonauta envolvido na ação do miRNA, mas em alguns casos é possível encontrar a participação de AGO7 (ENDO; IWAKAWA; TOMARI, 2013) e AGO2 (ZHANG et al., 2011).

A grande maioria dos genes do miRNA é específica da espécie ou família, sugerindo rápida evolução (CUPERUS; FAHLGREN; CARRINGTON, 2011; FAHLGREN et al., 2007). A sequência do miRNA maduro, composta por 21-24 nucleotídeos, tende a ser bastante conservada, pois é a que dá especificidade, e é utilizada para classificar os miRNAs em famílias com base na sua sequência, os miRNAs são agrupados em famílias compostas por diferentes membros designados por uma letra após o número do miRNA (por exemplo, miR398a, miR398b).

Em *A. thaliana* quatro miRNA foram até então identificados e relacionados com a homeostase de cobre: miRNA397, miRNA398, miRNA408, miRNA857, sendo os três primeiros altamente conservado em outras plantas, sugerindo um papel importante na regulação da concentração de cobre na planta (BURKHEAD et al., 2009; PILON, 2017). Em *A. thaliana* a família miRNA397 possui dois genes, miRNA397a e miRNA397b, ambos sendo expressos em diferentes órgãos da parte aérea, com maior expressão no caule, semente e pecíolo e menor expressão nas folhas (WANG et al., 2014). A família miRNA397 tem as lacases como alvo. Em plantas de *A. thaliana* nas quais foram superexpressos membros da família miRNA397, em condição de controle comparadas a selvagem (Col-0) foi observado uma redução do tamanho da planta, redução na produção de lignina e também menor rendimento de sementes (WANG et al., 2014). Diferentemente, em *O. sativa*, plantas mutantes que tiveram o gene OsmiRNA397 superexpresso, apresentaram maior número de panículas, além de aumentar o número de sementes e o peso das mesmas (ZHANG et al., 2013).

A família miRNA398 possui três genes, que codificam para miR398a, miR398b, miR398c (JONES-RHOADES; BARTEL, 2004). AtmiRNA398a e AtmiRNA398b se diferenciam por 1 nucleotídeo em sua estrutura (SUNKAR; ZHU, 2004). É sugerido que AtmiRNA398a e AtmiRNA398c possuem como alvo Cu/Zn-SOD (PILON, 2017). A transcrição dos genes miRNA398b e miRNA398c são ativadas por SPL7 quando em deficiência de Cu, enquanto AtmiRNA398a não tem sua expressão modificada em diferentes concentrações de cobre (ABDEL-GHANY; PILON, 2008; ARAKI et al., 2018). Já o miRNA408 é codificado por apenas um gene e possuem como alvos as lacases, principalmente LAC3, LAC12 e LAC13, e também plastocianinas e cupredoxina (ABDEL-GHANY; PILON, 2008). A superexpressão de miRNA408 em *O. sativa* aumentou o número de ramificações e aumento do peso dos grãos (ZHANG et al., 2017).

1.6 TRABALHO PRECURSOR

De acordo com trabalho anterior a este, realizado por Del Frari (DEL FRARI, 2018), utilizando o mesmo o mutante *oshma5* em condições de deficiência de Cu e excesso, foi possível verificar que o mutante *oshma5* apresentou redução nos componentes morfológicos analisados em comparação ao tipo selvagem (cv. Nipponbare), assim como alterações bioquímicas e na concentração de nutrientes. Em adição, foi observado no tipo selvagem uma maior expressão de miRNAs (397ab, 398b e 408) quando em deficiência de Cu em comparação as plantas em controle. Porém, não foram avaliadas a expressão dos mesmos no mutante *oshma5*, assim como não foi avaliada a expressão de diversos genes possivelmente envolvidos na economia de Cu, e também genes que possam ter algum envolvimento na partição de outros elementos (Fe, Zn e Mn). Assim, em complementação ao trabalho de Del Frari (DEL FRARI, 2018), nosso trabalho teve foco nas alterações morfológicas, moleculares e na partição de nutrientes.

1.7 OBJETIVOS

1.7.1 GERAL

Caracterizar os mecanismos moleculares envolvidos na economia de cobre em plantas de arroz selvagem e mutante *oshma5* e a interação com outros micronutrientes

1.7.2 ESPECÍFICOS

1- Verificar a concentração dos elementos Cu, Fe, Zn e Mn nas raízes e parte aérea em planta mutante *oshma5* e selvagem (WT) em condições de controle (CC) e deficiência de cobre (-Cu);

2- Avaliar a expressão relativa de genes relacionados a homeostase de cobre;

3- Avaliar a expressão relativa de genes de absorção e armazenamento de Ferro;

ARTICLE

The copper economy response in rice (*Oryza sativa* L.) is partially conserved

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ABSTRACT

Copper (Cu) is an essential nutrient for plants, and its deficiency leads to several negative effects, which consequently influence important physiological processes such as photosynthesis. Therefore, understanding the mechanisms controlling copper homeostasis in plants is key for engineering plants for increased biomass production and yield. Cu is necessary for plastocyanin function, since it requires a Cu atom for proper function in electron transfer reactions at thylakoid membranes. In the model plant *Arabidopsis thaliana*, Cu deficiency leads to Cu economy, in which plants prioritize Cu usage by plastocyanin in detriment of non-essential proteins that use Cu as a cofactor. OsHMA5 is a Cu xylem loading transporter, and its loss-of-function leads to decreased Cu translocation from roots to shoots. Here we aimed at understanding how rice (*Oryza sativa* L.) plants respond to Cu deficiency. For that, we use *oshma5* mutant rice plants and its wild type cv. Nipponbare (WT) in two contrasting condition (CC, Control- Condition; -Cu, Copper deficiency condition) as tools to uncover the changes driven by copper deficiency. We found that *oshma5* plants are consistently smaller than WT, regardless of being grown under control or Cu deficiency. We also observed that *oshma5* plants have increased Cu concentration in roots in both treatments, decreased Cu concentration in shoots under Cu deficiency, and therefore decreased Cu translocation index, compared to WT. We also found that *oshma5* plants Fe shoot concentration increases drastically under Cu deficiency, indicating an interplay with Fe homeostasis. Gene expression analysis revealed that OsCOPT1 and OsCOPT5 in shoots and root were up-regulated under –Cu in both genotypes. In shoots of WT, OsCSD1 were up-regulated, while OsCSD3 and OsCSD4 were down-regulated under –Cu. In contrast, OsCSDs in shoots of *oshma5* were not regulated, but show generally lower expression levels compared to WT, suggesting that *oshma5* in CC may be already under mild Cu deficiency. In roots, OsCSD2 were down-regulated under –Cu, and OsCSD1, OsCSD3,

OsCSD4 were not regulated under –Cu in WT plants. In roots of *oshma5,* OsCSD1, OsCSD2, OsCSD3 were up-regulated and OsCSD4 down-regulated under –Cu. OsFSD1 in shoots were down-regulated in WT and up-regulated in *oshma5* under –Cu. OsFSD2 in shoots were down-regulated under –Cu. In roots, OsFSD2 did not change gene expression in WT under –Cu, while *oshma5* were up-regulated in –Cu. OsmiRNAs in shoots and roots were up-regulated under –Cu in both genotypes. We also found some changes in Fe homeostasis genes, which may be related to the increase in Fe uptake found in *oshma5* plants. These results are the first report that rice (*Oryza sativa* L.) may have a conserved Cu economy mechanism.

Keywords: Copper homeostasis, Cu-miRNAs, gene expression, OsHMA5, *Oryza sativa*

2 INTRODUCTION

Copper (Cu) is an essential micronutrient for growth and development of plants. Cu can act as a cofactor of several proteins that are involved in fundamental physiological processes such as photosynthesis and respiration, mainly in electron transport chains. Symptoms associated with Cu deficiency are reduced biomass and growth, chlorosis in young leaves, reduced photosynthetic activity and pollen fertility (HUANG et al., 2016; YRUELA, 2013). To maintain the Cu homeostasis in plant cells, several processes have to be tightly regulated, such as the absorption of Cu by roots, transport from roots to shoots, and correct distribution among organs, cells and organelles (BURKHEAD et al., 2009; MARSCHNER, 2012; PANDEY, 2018; YRUELA, 2009).

In the model species *Arabidopsis thaliana*, Cu deficiency leads to the so-called "Cu economy" response, which allows preferential use of Cu by plastocyanin by decreasing expression of non-essential cupric proteins (BURKHEAD et al., 2009; PRINTZ et al., 2016). Cu economy is regulated by the SPL7 transcription factor (*SQUAMOSA promoter binding like protein* 7). SPL7 is constitutively expressed, and locally up regulates its target genes: (1) high-affinity Cu transporters of the COPT/CTR family; (2) Fe Superoxide dismutase (FSDs); and (3) miRNA that suppress cupric proteins such as Cu/Zn Superoxide Dismutase (CSDs) and Laccases (PRINTZ et al., 2016). Under Cu deficiency, *A. thaliana* plants decrease use of CSDs, increase use of FSDs and up regulate the uptake of Cu from the soil, with the goal of protecting photosynthesis. However, it is not known if the Cu economy response is conserved in other species.

Rice (*Oryza sativa*) is the model species for monocots, and also an important agricultural crop and a staple food in the world. Different transporters and proteins linked to Cu homeostasis in rice have been described (HUANG et al., 2016; MIGOCKA, 2015; PRINTZ et al., 2016; PUIG, 2014). The CTR-like/COPT family in rice has seven members (OsCOPT1-7) (YUAN et al., 2011), and OsCOPT1, OsCOPT5 and OsCOPT7 were shown to be up regulated under Cu deficiency in roots and shoots (YUAN et al., 2011). The SOD gene family has also been described, with four genes coding for CuZnSOD (OsCSD1, OsCSD2, OsCSD3, OsCSD4), two genes for FeSOD (OsFSD1, OsFSD2) and two genes for MnSOD (OsMnSD1, OsMnSD2) (GILL et al., 2015; GILL; TUTEJA, 2010; NATH et al., 2014). Moreover, rice has homologous miRNAs from families AtmiRNA397 (PILON, 2017), AtmiRNA398 and AtmiRNA408 (PILON,

2017; ZHANG et al., 2017), linked to Cu economy in *A. thaliana*, and shown to target and regulate Cu-protein expression linked to Cu economy. AtmiRNA397 regulate the expression of laccase family members, AtmiRNA398 Cu/Zn-SOD and Cu-chaperone, and AtmiRNA408 some members of laccase family, phytocyanins and cupredoxins (PILON, 2017; SHAHBAZ; PILON, 2019; ZHANG et al., 2017).Whether these genes are concertedly regulated by Cu deficiency in rice was not yet described.

Another protein family involved in Cu homeostasis are the P_{1B}-ATPases subfamily (AXELSEN; PALMGREN, 1998; MIGOCKA, 2015), also known as HMA (*Heavy Metal-Associated*). HMAs are proteins that contribute to intracellular transport between organelles and cytosol, Cu detoxification, as well as Cu efflux to the extracellular environment (MIGOCKA, 2015). In rice there are nine genes that code for P1B-ATPases (OsHMA1-9) proteins, with a subset involved in Cu transport (MIGOCKA, 2015; WILLIAMS; MILLS, 2005). Among these, OsHMA5 is a plasma membrane transporter, which performs Cu efflux into the xylem from pericycle cells. OsHMA5 loss of function mutants show reduced Cu translocation from roots to shoots and seeds, and increased Cu concentration in roots (DENG et al., 2013). Therefore, shoots of *oshma5* plants are likely to have some extent of Cu deficiency even under Cu-replete conditions. However, there is no data regarding how *oshma5* plants regulate Cu homeostasis.

Here we focused on the Cu deficiency response, and whether there is a Cu economy response in rice. Our findings suggest that rice has a partially conserved mechanism of Cu economy compared to *A. thaliana*, and changes in the external Cu concentration may implicate in deregulation of others metal elements concentration in plant organs. Our data shows for the first time the integrated Cu deficiency response in rice, which allows understanding how rice plants regulate Cu homeostasis. In addition, this knowledge can be used to obtain modified plants with a change in Cu concentration in edible plant organs, which may imply in reducing or increasing Cu entry in the food chain as needed (HUANG et al., 2016; YRUELA, 2005, 2009).

2.1 METHODS

2.1.1 PLANT MATERIAL AND GROWN CONDITION

Seeds of *oshma5* mutant and wild type (WT) cv. Nipponbare plants were disinfected with sodium hypochlorite 2% for 10 min, washed with deionized water five times, and hydro-primed for 24h in the dark at 25 °C. The *oshma5* (NF8524) rice mutant, generated by retrotransposon *Tos17* insertion in the second exon of OsHMA5 gene, was obtained from NIAS, and was characterized previously (DENG et al., 2013). Seeds were germinated for 8 days at 25°C on filter paper soaked with deionized water. Seedlings were transferred to a Styrofoam holder oven a 400 mL dark plastic cup (4 seedlings per cup) containing deionized water for 7 days. Seedlings were transferred to a modified nutritive solution for 15 days containing 0,7 mM K_2SO_4, 0,1 mM KCl, 0,14 mM KH_2PO_4, 1,39 mM $Ca(NO_3)_2$.$4H_2O$, 0,244 mM $MgSO_4.7H_2O$, 10 µM H_3BO_3, 0,5 µM $MnSO_4$. H_2O, 0,2 µM $CuSO_4$. $5H_2O$, 0,275 µM $ZnSO_4.7H_2O$, 0,05 µM Na_2MoO_4. $2H_2O$, e 0,1 mM Fe^{+3}–EDTA (ISHIMARU et al., 2005). To induce Cu deficiency, $CuSO_4.5H_2O$ was omitted from the solution.

2.1.2 GROWTH MEASUREMENT

To determine plant growth under control and Cu deficiency, shoot and roots from individual plants after 15 days of treatment were measure using a common plastic ruler. Means were obtained from 12 plants.

2.1.3 DETERMINATION OF Cu, Fe, Zn AND Mn IN SHOOT AND ROOTS RICE PLANTS, AND TRANSLOCATION INDEX CALCULATION

Samples of roots and shoot from plants treated for 15 days (30 days old) were air dried in 65°C for 72h. Samples (n=4, pools of 6 plants) were grounded to powder and then 0.5 g was digested in 10 mL of HNO_3+ $HClO_4$ (3:1 v/v), pre-digestion at 80°C for 1h, 150 °C for 5h. The clear digested were diluted to 20 mL with deionized water. A flame atomic absorption spectrometry instrument (Perkin Elmer, A- Analyst 200) was used to measure Cu, Fe, Zn and Mn. Relative translocation index (TI%) Root-to-shoot was calculated according to the following formula (GALAL; SHEHATA, 2015):

$$\text{Tranlocation index (TI\%)} = \frac{\text{Metal Concetration in shoot}}{\text{Metal Concentration in Root}} \text{X } 100$$

2.1.4 RNA EXTRACTIONS, CDNA SYNTHESIS AND GENE EXPRESSION BY RT-QPCR

Shoots and root from of *oshma5* rice mutant and WT were collected after 15 days of treatment (30 days old). Samples (50-100 µg) composed of four biological replicate containing a pool of 3 plants were grounded to powder with liquid nitrogen and extract using Concert Plant RNA Reagent (Invitrogen®, Carlsbad, CA, USA). RNA quantification was performed with NanoDrop® (Thermo Fisher Scientific, Waltham, USA). RNA (2 µg) was treated using DNase I (Invitrogen®, Carlsbad, CA, USA). First-strand cDNA was performed with OligodT and reverse transcriptase (M-MLV, Invitrogen®, Carlsbad, CA, USA). RT-qPCR reaction was performed in a final volume of 20 µL composed of 10 µL of cDNA samples diluted 50 times, and 10 µL mix reaction containing: 2 µL of 10 × PCR buffer, 1.2 µL of 50 mM $MgCl_2$, 0.2 µL of 10 mM dNTPs, 0.4 µL of forward primer (10 pM), 0.4 µL of reverse primer (10 pM), 3.75 µL of water, 2 µL of SYBR green (1:10,000 Molecular Probe), and 0.05 µL of Platinum Taq DNA Polymerase (5 U μL^{-1}, Invitrogen, Carlsbad, CA, USA). Reaction settings were 5 min at 94 °C, followed by 40 cycles of 10 s at 94 °C, 15 s at 60 °C, 15 s at 72 °C and 40 s at 60 °C for fluorescence data collection, using a StepOne Real-Time Cycler (Applied Biosystems, Foster City, USA). All primers used are listed in Table 1. Individual PCR efficiency was obtained with LinReg software (RAMAKERS et al., 2003) for the relative gene expression calculation (RICACHENEVSKY et al., 2011).

2.1.5 STATISTICAL ANALYSIS

Mean values from biological replicate were compared by Student's t test using $p<0.05$, in GraphPad Prism 7 software.

2.2 RESULTS

2.2.1 OSHMA5 IS IMPORTANT FOR PROPER PLANT GROWN

To investigate plant growth under Cu deficiency, plants from wild type (WT) and mutant *oshma5* rice were cultivated under control (CC) and in Cu deficiency (-Cu) for 15 days. *oshma5* plants showed slightly decreased growth, regardless of Cu treatment, compared to WT (Figure 1a). Shoot length of *oshma5* plants was also reduced under –Cu when compared to control (Figure 1a). Curiously, *oshma5* showed increased root growth under -Cu (Figure 1b). Therefore, our data show that lack of functional OsHMA5 and presumably altered Cu distribution results in changes in biomass partitioning in rice plants.

2.2.2 COPPER DEFICIENCY MODIFIED Fe, Zn AND Mn PARTITION IN PLANT

To understand how the loss of function of OsHMA5 change elemental composition, we measured the concentration of elements in roots and shoots of plants under control (CC) and copper deficiency (-Cu) condition. Cu concentration in shoots was similar in WT plants regardless of Cu treatment. For *oshma5*, however, shoots showed decreased Cu concentration when exposed to –Cu, compared to *oshma5* plants in control conditions as well as compared to WT in the same condition (Figure 2a). On the other hand, *oshma5* roots showed increased Cu concentration compared to WT in both CC and –Cu. Moreover, both genotypes showed decreased Cu concentration in roots of plants under –Cu compared to plants of the same genotype in control conditions (Figure 2b). These results confirm that OsHMA5 is involved in Cu translocation from roots to shoots (DENG et al., 2013). Our data also suggest that *oshma5* plants are more likely to present -Cu responses.

Fe concentration in shoots of WT plants was not changed by -Cu (Figure 2c). However, *oshma5* plants under –Cu condition showed ~4 fold increase in Fe shoot concentration compared to WT as well as to *oshma5* plants under control conditions (Figure 2c). Roots from WT plants showed reduced Fe concentration in –Cu condition, while *oshma5* roots increases root Fe concentration under –Cu condition compared to control (Figure 2d).

Mn shoot concentration was increased in WT plants in –Cu condition compared to control. However, *oshma5* plants showed higher Mn concentration in both conditions compared to WT (Figure 2e). In roots, both WT and *oshma5* plants showed increased Mn concentration

under –Cu condition, but no difference was found between genotypes (Figure 2f). Shoot Zn concentrations were nearly unchanged, while root concentration were decreased by –Cu in WT but increased in *oshma5* (Figure 2g and 2h). Under –Cu *oshma5* showed higher Zn concentration in roots compared to WT plants (Figure 2h). Moreover, we found that seed concentration of Cu was decreased in *oshma5*, while Fe concentration was clearly increased (Figure 2i). Taken together, these data showed that OsHMA5 is important for Cu homeostasis, and suggest that perturbation in Cu root to shoot translocation changes homeostasis of other elements.

In order to better visualize the translocation capacity of each genotype for all measured elements, we calculated the relative translocation index (TI%). As expected, we observed that WT plants have higher Cu translocation index in both growth conditions compared to *oshma5*, evidencing the effect of OsHMA5 loss of function already described (DENG et al. 2015). Interestingly, WT plants increased their Cu translocation under –Cu, whereas *oshma5* plants showed no difference (Figure 3a). We also found that, while WT plants slightly increase their Fe translocation under -Cu, *oshma5* show much higher translocation compared to WT (Figure 3b). For Mn, *oshma5* plants showed translocation index reduction under -Cu compared to CC condition, while WT the translocation index were not changed by –Cu (Figure 3c). Moreover, WT plant showed an increase on Zn translocation index under –Cu condition compared to the control condition, whilst *oshma5* had a reduction under –Cu condition compared to *oshma5* under control and WT under –Cu condition (Figure 3d).

2.2.3 COPT GENES ARE INDUCED UNDER CU DEFICIENCY

Our data supports that shoots of *oshma5* plants are more prone to -Cu due to decrease Cu translocation. Therefore, it is reasonable to suggest that *oshma5* plants, even under CC conditions, may have already undergone changes normally associated with -Cu, i.e., they may already be in Cu economy mode. In order to determine whether this is the case, we aimed at analyzing the expression of genes putatively involved in Cu economy in both genotypes. First, we analyzed OsCOPT genes, which were already shown to be up regulated by –Cu (YUAN et al., 2011). In shoots, OsCOPT1 was up regulated under –Cu condition in WT and *oshma5*, and to a higher extent in *oshma5* (Figure 4a). Similarly, OsCOPT5 was also up regulated in shoots of both genotypes by –Cu (Figure 4b). In roots, OsCOPT1 was up-regulated by –Cu in WT plants, but not in *oshma5* (Figure 4c). OsCOPT5 was also shown to be up regulated in both genotypes

under -Cu. We also analyzed OsCOPT7 gene expression, but found no expression in our experimental conditions. Therefore, we conclude that the expression of OsCOPT1 and OsCOPT5 was up regulated under -Cu in both organs, corroborating previous results (YUAN et al., 2011) and confirming that our treatment led to –Cu in both genotypes.

2.2.4 CU/ZN SUPEROXIDE DISMUTASE GENES ARE AT LEAST PARTIALLY DEPENDENT ON Cu CONCENTRATIONS IN ROOTS AND SHOOTS

Given that Cu/Zn superoxide dismutase (CSD) are repressed in *A. thaliana* under -Cu as part of the Cu economy response, we sought to analyze CSD gene expression. The rice genome has four CSD genes (GILL et al., 2015). In shoots, we found that CSD expression in general is lower in *oshma5* compared to WT, regardless of treatment, suggesting that the lower Cu translocation leads to lower total CSD expression in shoots (Figure 5). OsCSD1 was up regulated by –Cu in WT, but not in *oshma5* (Figure 5a). OsCSD2 and OsCSD3 showed similar patterns, with lower expression in *oshma5* compared to WT in both conditions, and a slight down-regulation of OsCSD3 in shoots of WT plants (Figure 5b and 5c). Interestingly, OsCSD4 was clearly down-regulated by –Cu in WT, but was already low in shoots of *oshma5* plants under CC conditions. Taken together, these results suggest that shoots from *oshma5* are already acclimating to low Cu conditions (Figure 5d).

In roots, we found that most OsCSDs are not regulated by –Cu in WT plants (Figure 5e and 5h). The only exception is OsCSD2, which is down-regulated (Figure 5f). However, in *oshma5*, we found that OsCSD1, OsCSD2 and OsCSD3 are up-regulated under –Cu (Figure 5e and 5g), while OsCSD4 is down-regulated (Figure 5h). Therefore, our data indicate that lack of a functional OsHMA5 resulted in deregulation of Cu homeostasis in both shoots and roots of rice plants, and that expression of CSDs is at least partially dependent on Cu concentration in roots and shoots.

2.2.5 IRON SUPEROXIDE DISMUTASE (FSD) ARE DIFFERENTLY EXPRESSED UNDER Cu CONCENTRATION AND ORGAN SPECIFICITY

We also evaluated gene expression of two superoxide dismutase genes that use Fe as cofactor (FSD), and which are also part of the Cu economy response in *A. thaliana* (PRINTZ et al., 2016). Contrary to what was expected, we found that both OsFSD1 and OsFSD2 are down-

regulated by –Cu in WT shoots (Figure 6a and 6b). However, in shoots *oshma5*, we found that OsFSD1 had lower expression in control condition compared to WT, and was up-regulated by –Cu (Figure 6a). OsFSD2, on the other hand, was down regulated by –Cu treatment in shoots of *oshma5* plants (Figure 6b). In roots, we only detected OsFSD2 expression for both genotypes, and found that whereas OsFSD2 expression is slightly up regulated by –Cu treatment in WT plants, up to 2-fold increase is found in *oshma5* plants (Figure 6c). Therefore, OsFSD2 expression seems to be important for *oshma5* plants exposed to –Cu conditions. This indicates that OsFSDs does not seem to have the same role in Cu economy responses as found in *A. thaliana* in rice shoots, although Fe concentration increases dramatically in *oshma5* shoots. In roots, OsFSD2 appear to have a role under –Cu, together with Fe concentration increase in *oshma5* roots of plants under –Cu.

2.2.6 miRNAS ARE INDUCED UNDER COPPER DEFICIENCY

MicroRNAs are an important class of non-coding RNA that are responsible for post-transcriptional regulation. We analyzed four homologous miRNAs from *A. thaliana* that are involved in Cu economy mechanism (PRINTZ et al., 2016). In shoots, we observed that in both WT as *oshma5* shoots, OsmiRNA397ab is dramatically up regulated in -Cu compared to CC condition (Figure 7a), but no difference was found comparing genotypes under –Cu and CC. On the other hand, OsmiRNA398b in *oshma5* was up regulated compared to WT under –Cu, while *oshma5* had lower expression under CC (Figure 7b). OsmiRNA408 was up-regulated under –Cu condition in *oshma5* compared to WT under same condition (Figure 7c).

In roots, we found little difference between the genotypes in CC condition. However,–Cu up regulated all miRNA tested (Figure 7d, 7e and 7f). For OsmiRNA397ab and OsmiRNA398b *oshma5* showed no difference expression compared WT under –Cu (Figure 7d and 7e). Nevertheless, OsmiRNA408 in *oshma5* showed less expression under –Cu compared to WT (Figure 7f). We also analyzed OsmiRNA398a gene expression, but found no expression in our conditions. These results suggest that OsmiRNAs in *oshma5* up-regulation under –Cu were led to low Cu root-shoot translocation in comparison to CC.

2.2.7 COPPER CHAPERONE

We analyzed one Cu chaperone (OsCCS), we observed that under –Cu condition shoots of WT were up-regulated, we did not observed expression changes in *oshma5* plants (Figure 8). Were also analyzed gene expression in roots, but no expression was found in our conditions (Figure 8).

2.2.8 IRON UPTAKE AND STORAGE GENES

We also analyzed the relative expression of genes involved in Fe absorption and vacuolar storage as we observe high Fe concentration in the plant organs, especially in *oshma5*. In shoots, OsYSL15 were down-regulated under –Cu in WT shoots, no difference were found between genotypes under –Cu (Figure 9a). OsNRAMP1 and OsIRO2 in WT were also downregulated under –Cu, but not in *oshma5,* and under CC, *oshma5* had lower expression in comparison to WT (Figure 9b and 9c). Under –Cu, *oshma5* showed lower expression than WT, while no difference was found when comparing the two conditions (Figure 9d). OsVIT2 was up-regulated under –Cu condition in WT plants compared to CC, but not in *oshma5* in both condition (Figure 9e). However, *oshma5* had higher expression in CC and lower expression in –Cu in comparison to WT plants (Figure 9e).

In root, we found that most Fe-related genes were not regulated by -Cu in WT. However, in oshma5 plants we found that OsYSL15, OsNRAMP1 and OsIRO2 were up-regulated, while OsIRT1 was down regulated. OsVIT2 was up-regulated in both genotypes by –Cu. These results suggest that -Cu may be modulating Fe related genes.

2.3 DISCUSSION

Here we use a mutant plant of the gene HMA5 (*oshma*5) and its respective wild type *Oryza sativa* cv. Nipponbare (WT). We observed that in control condition the mutant plants (*oshma*5) already had reduction on the shoot length when compared to the WT plants (Figure 1A). Therefore, when the genotypes were grown in a -Cu condition, the *oshma5* mutant plants had a lower shoot growth compared to WT, implying that decreased Cu translocation to shoots may negatively affecting photosystem functionality (YRUELA, 2005, 2009). However, we found

the opposite in roots of *oshma5* where were an length increase under -Cu, suggesting a mechanism for copper uptake involved increased roots growth (GRUBER et al., 2013).

We also confirmed that Cu is less translocated to shoots in the absence of a functional copy of OsHMA5 (DENG et al., 2013). Interestingly, we found that –Cu treatment in WT plant increases Cu translocation, whereas *oshma5* are not able to increase their translocation, indicating that this ability is dependent on OsHMA5. We also found changes in the concentrations of other elements such as Mn and Zn. Mn concentration in WT shoot and roots under –Cu were increased (Figure 2e and 2f) but had the same TI% (Figure 3c). Shoots of *oshma5* was not changed the Mn concentration between conditions, and roots showed a reduction in Mn concentration under –Cu (Figure 2g and 2h), and lower TI% under –Cu (Figure 3c). In addition, Zn concentration in shoots were not affect by exogenous Cu concentration (Figure 2g); while in roots, WT showed a reduction and *oshma5* an increase of Zn under –Cu (Figure 2h). Therefore, these results are partially in agreement with other studies that changes in Cu availability slightly affect Mn and Zn concentration and translocation (ANWAR; TAWAB; BHUTTO, 2016; LI et al., 2019).

An even more pronounced increase in Fe concentrations in the mutant under -Cu condition was observed (Figure 2 c-h). This change may be linked to the already known cross talk between iron and copper (PÄTSIKKÄ et al., 2002; PEREA-GARCÍA et al., 2013), but which is yet poorly described in rice (ANDRÉS-BORDERÍA et al., 2017). Another possibility the high Fe concentration (Figure 2c) in TI% (Figure 3b) might be a plant strategy to supply the demand of Cu with a similar potential redox metal in order to minimize the harmful effects, as it happens in the copper economy system described in *A. thaliana* (BURKHEAD et al., 2009; PEÑARRUBIA et al., 2015).

To gain more clarity about the concentrations of the elements in the shoots and in the roots, mainly Cu, we analyzed the expression of two transporters of high affinity of copper of the family CTR/COPT (PUIG, 2014). Plants under copper starvation showed increased expression of OsCOPT1 and OsCOPT5 genes both shoot and roots (Figure 4), this effect has already been described in other studies (YUAN et al., 2010, 2011). These transporters are highly expressed under -Cu (ANDRÉS-BORDERÍA et al., 2017; BURKHEAD et al., 2009). In the model plant *A. thaliana* AtCOPT1 is located in plasma membrane and responsible for Cu uptake from external medium (SANCENÓN et al., 2004), whereas AtCOPT5 is at an internal membrane, responsible

for copper delivery from vacuole to cytosol under -Cu (CARRIÓ-SEGUÍ et al., 2019; GARCIA-MOLINA et al., 2011; KLAUMANN et al., 2011). Therefore, since OsCOPT1 and OsCOPT5 genes were highly up-regulated under -Cu, we may suggest that they can be considered as indicators of -Cu genes in rice.

We analyzed expression of genes involved in the detoxification of free radicals. SOD enzymes are considered among the most effective of the antioxidant system in reducing ROS (GILL; TUTEJA, 2010), which are responsible for converting O_2-* (superoxide anion) into H_2O_2 and O_2, thus reducing the possibility of the Haber-Weiss reaction, since O2*-- and H_2O_2 are substrates for this reaction (GILL et al., 2015; GILL; TUTEJA, 2010). In foliar tissues, under -Cu and control conditions, the *oshma5* mutant showed lower expression of SODs (OsCSD2, OsCSD3 and OsCSD4) enzymes compared to the wild type (Figure 5 b-d), which can be response to changes in physiological processes (PEÑARRUBIA et al., 2015; RAVET; PILON, 2013; YRUELA, 2013). An increase in gene expression of these enzymes is triggered under biotic and abiotic disorder, with superoxide dismutase as the first line of defense (GILL; TUTEJA, 2010). As described in *A. thaliana*, when there is a low concentration of copper in planta result in so far described mechanism called copper economy (BURKHEAD et al., 2009; YAMASAKI; PILON; SHIKANAI, 2008). In one of the stages of this mechanism there is the prioritization of allocating copper to more essential proteins. In that context, the Cu/Zn-SODs can have their expression reduced. In our experiment, Cu/Zn-SOD gene expression was lower in the leaves of *oshma5*. This result suggests that *oshma5* plants under control condition were already presenting signals of -Cu in shoots (KAMINAKA et al., 1997; KANEMATSU; ASADA, 1989; PRAKASH SANYAL et al., 2018; UEDA et al., 2013).

Other members of SOD family are the FeSODs, which use iron as cofactor. The first expression of this enzyme was mentioned in the 80s decade, when this metalloenzyme was not yet described in higher plants (PAN; HWANG; LIU, 1999). Today we known that there are two functional isoenzymes in rice: FeSOD1 and FeSOD2 (BHOOMIKA; PYNGROPE; DUBEY, 2013; FENG et al., 2005). We decided to verify their expression in this study for being described in *A. thaliana* as increased expression for ROS detoxification mechanism, whereas Cu/Zn-SOD has its expression reduced in -Cu conditions (ABDEL-GHANY et al., 2005a). Our data show that under -Cu condition *oshma5* mutant plants genes for OsFeSOD1 and OsFeSOD2 were highly up regulated in shoots and OsFeSOD2 in root compared to WT plants, but not expression

were found under our condition for expression of FeSOD1 in roots of WT and *Oshma5* (Figure 6).

Changes in the gene expression of SODs in -Cu condition were described in *A. thaliana* (BURKHEAD et al., 2009; PEÑARRUBIA et al., 2015) and include the post-transcriptional regulation of copper proteins via specific miRNAs (ARAKI et al., 2018; YAMASAKI et al., 2009). MicroRNAs (miRNAs) that are regulated by copper availability and modulate proteins by bind in copper protein mRNAs (PILON, 2017). We evaluated four Cu-miRNAs (miR397ab, miR398a, miR398b and miR408). We found that -Cu modulated the expression in three MiRNAs in rice (Figure 7). It is suggested that MiRNA398b is responsible for its regulation of CSD2 and CCS in *A. thaliana* (ABDEL-GHANY; PILON, 2008; SHAHBAZ; PILON, 2019) and in *Populus trichocarpa* (RAVET et al., 2011). In Arabidopsis, MiRNA397 specifically target laccases (LAC2, LAC4 and LAC17) (ABDEL-GHANY; PILON, 2008; LIU et al., 2017; WANG et al., 2014; YAMASAKI et al., 2009). In rice, MiR397ab is known to reduce *OsLAC* expression, (ZHANG et al., 2013). In this study, it was observed that MiRNA397ab showed higher expression under -Cu, so it suggest that may be acting on the reduction of Cu consumption by down regulation of specific cupric proteins (BURKHEAD et al., 2009). In this sense, miRNA408 also showed higher expression under -Cu , which is responsible for the regulation of *LAC13* and *ARPN* (plancyanine) transcripts in Arabidopsis (ABDEL-GHANY; PILON, 2008; SHAHBAZ; PILON, 2019; ZHANG; LI, 2013). Interestingly, CCS (copper chaperone for superoxide dismutase) were up regulated in shoots of WT plants under –Cu (Figure 8), this result is contradictory since miRNA398 could target the CCS mRNA (BLABY-HAAS et al., 2014; TAPKEN et al., 2015). We suggest that this up regulation in gene expression of MiRNAs under -Cu condition might be also conserved in *Oryza sativa* (Figure 7).

To evaluate whether the Fe genes are regulating the Fe uptake and distribution we analyzed OsYSL15, OsNRAMP1, OsIRO2, OsIRT1 and OsVIT2 genes. OsYSL15 is a plasma membrane transporter, under Fe deficiency is up-regulated in all tissues and down-regulated under high Fe concentrations (LEE et al., 2009). In our study, we observed that OsYSL15 was down-regulated in WT under -Cu, while in *oshma5* shoots the expression of OsYSL15 was not changed, but in *oshma5* roots was up-regulated (Figure 2a and 2f). These data contrast with the expression of this gene in -Cu rice plants in another study, in which it was not possible to detect the OsYSL15 expression in leaves and roots (INOUE et al., 2009). Interestingly, OsYSL15

uptake and transports of Fe is bound to deoxymugeinic acid (INOUE et al., 2009). This increase in Fe concentration *oshma5* shoots (Figure 2c) might be due to the up-regulation of OsYSL15 in -Cu. OsNRAMP1 in yeast complementation assays was able to transport Fe and Mn, and located in the plasma membrane (TAKAHASHI et al., 2009). With the fusion with GFP, OsNRAMP1 has its greatest expression in the roots, mainly in the epidermis, endodermis and pericycle, suggesting also its function in xylem loading of Fe and Cd (BASHIR; SEKI; NISHIZAWA, 2019; CAILLIATTE et al., 2010; TAKAHASHI et al., 2009). However, it seems that in *A. thaliana* plants AtNRAMP1 expression is more selective, when AtNRAMP1 knockout plant were submitted to high concentrations of Fe the plant changed the homeostasis of Mn but not Fe (CAILLIATTE et al., 2010). This can be observed in our results, there was higher concentration of Mn in the roots of both genotypes (Figure 2d and 2e). However, there were no changes in TI% of WT, and a reduction in TI% of *oshma5* (Figure 3c). Interestingly, rice plants that were stressed with As and Cd showed an increase in the concentration of Mn in the roots and shoot, while there was no change in the concentration of Fe in the tissues (TIWARI et al., 2014).

The OsIRO2 transcription factor is linked to gene regulation under Fe deficiency (OGO et al., 2007). In our experiment, we observed a higher Fe concentration in *oshma5* shoots in comparison to WT, while in *oshma5* roots had lower concentration in CC and higher concentration in -Cu (Figure 2c and d). However, when we checked the expression of OsIRO2 we found that was highly expressed in WT shoots under CC and down-regulation in –Cu (Figure 9c and h). In the roots, there was no difference between the genotypes in our conditions but there was an increase in *oshma5* under -Cu. Under Fe-sufficient conditions, OsIRO2 is down-regutated (MASUDA et al., 2019; OGO et al., 2007), suggesting that up regultation of OsIRO2 in *oshma5* plants under –Cu might not related to Fe concentration. Interestingly, *osiro2* knockout plants showed increased tolerance to Fe deficiency, with higher grain yield and higher Fe concentration in grains (MASUDA et al., 2017).

The expression of OsVIT2 (vacuolar iron transporter) is modulated by Fe concentration, down-regulated under Fe deficiency and up-regulated under excess (LIU et al., 2012; ZHANG et al., 2012). However, in our experiment we did not have a clear image of VIT2 expression, once in shoots was up-regulated under –Cu in WT, whilst in *oshma5* it was not altered (Figure 9e). In roots, in both genotypes OsVIT2 were up regulated under –Cu (Figure 9j), but contradictory with Fe content in WT (Figure 2 b and c).

2.4 CONCLUSION

The results showed that -Cu condition induced *oshma5* shoot reduction and increased root length. Cu deficiency modulated Fe, Zn and Mn partition and translocation in both genotypes. *oshma5* had an increase in Fe seed concentration and Cu reduction. *CDSs* were down and *FSDs* were up regulated under –Cu in *oshma5*. In general, both genotypes miRNAs were extremely up regulated. Exogenous copper concentration can modulated Fe uptake related genes. We show a first report of copper economy mechanism in rice.

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4 FIGURE LEGENDS

Figure 1. Shoot (a) and root (b) length in centimeters of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants. Plants with 15 days old were grown under control (CC) and Cu deficiency (-Cu) medium for more 15 days. Values are average ± standard error of 12 plants. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test [(*) p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 2. Element concentration of Shoot copper (Cu) (a) root copper (b), shoot iron (Fe) (c), root iron (d), shoot zinc (Zn) (e), root zinc (f), shoot manganese (Mn) (g), root manganese (h) and seeds (i) expressed in mg kg^{-1} dry weight of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. The analysis were performed using flame atomic absorption spectrometry instrument. Values are average ± standard error of n=4 pooled of 3 plants. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 3. Element translocation index (TI%) f rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. copper (a), iron (b), zinc (c) manganese (d). Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 4. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of OsCOPT1 from shoot (a) and root (c) and OsCOPT5 from shoot (b) and root (c) genes expression of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels.

Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 5. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of Cu-Zn superoxide dismutase; CSD1 from shoot (a) and root (e), CSD2 from shoot (b) and root (f), CSD3 from shoot (c) and root (g), CSD4 from shoot (d) and root (h) genes expression of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 6. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of Iron superoxide dismutase; FSD1 from shoot (a) and root was not dectable, FSD2 from shoot (b) and root (c) genes expression of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 7. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of MiRNA397ab shoots (a) and roots (d), MiRNA398b shoots (b) and roots (e), MiRNA408 shoots (c) and roots (f) genes expression of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu),

were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 8. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of OsCCS from shoots genes expression of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Figure 9. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of YSL15 shoots (a) and roots (f), NRAMP1 shoots (b) and roots (g), IRO2 shoots (c) and roots (h), IRT1 shoots d) and roots (i), VIT2 shoots (e) and roots (j) of rice (*Oryza sativa*) plants Nipponbare (WT) and mutant (*oshma5*) plants cultivated under control (CC) and Cu deficiency (-Cu) conditions. Relative expression values are average ± standard error of n=4 pooled of 3 plants and shown as relative value of housekeeper OsUBQ5 transcript levels. Asterisks indicate level of statistical difference between genotype plants grown under CC and –Cu condition, and between same condition (CC x CC or -Cu x -Cu), were used Student's t test[(*)p-value<0.05, (**)p-value<0.01, (***)p-value<0.001, (****)p-value<0.0001].

Table 1. Specific genes used in Reverse transcription quantitative real-time PCR analysis (RT-qPCR).

Genes	Forward (5'-3')	Reverse (5'-3')
OsUBQ5 (Housekeeper)	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT
OsCOPT1	CATGGGCGCCATGAAGTC	GTGAAGAGCACCTCCGAGTTCT
OsCOPT5	GCTGTCTCGCTCGTCATGGT	CGCACACACAAAACATCAACAA
OsCOPT7	GCCTAGGGTTTGGCTTTGC	ACAAGATCGGGAAACCAAACA
OsMiR397ab	GGCCTCATTGAGTGCAGCG	----
OsMiR398a	GGCGGTGTGTTCTCAGGTCA	----
OsMiR398b	GGCGGTGTGTTCTCAGGTCG	----
OsMiR408	CCGCTGCACTGCCTCTTC	----
OsMiRNA UNIVERSAL	-----	GTGCAGGGTCCGAGGT
OsCSD1	AGCTGTTGTTGTCCATGCTG	CTAACCCTGGAGTCCGATGA
OsCSD2	GGTGGCCATGAGCTTAGTCT	AAAAAGGGTGACATGGATGC
OsCSD3	AGGGGTGGTCATGAACTCAG	ATACCACCACCACCTCAAGC
OsCSD4	ATCGGACTTCAAGGCTGAAA	AAAGACGAAACGGCTAAGAGC
OsFSD1	GAGGCTTTTGTGAACCTTGG	GATTGCCTCACGGCTCAT
OsFSD2	GAGCTATGCCTCAGCAGGTC	GCTTACTTGGCTCCGTTGTC
OsYSL15	GGTGCGGGGATGATTTG	CCATACAAACTTGTCATGCTG
OsIRT1	ACTGGTGCCCATTCTGC	GCGAGGATGGGGATGG
OsIRO2	CGGATTTGGGAACAGGACA	GTTCCTGACGACTTTCTCCA
OsNRAMP1	CATGCTGCTCTACGTCGTC	CAGCTCACGACGAGACAC
OsVIT2	GGCTGCAGGCATCCAAGTAAATGT	CACTACAAGCACGCAGCAAACGTA

5 FIGURES

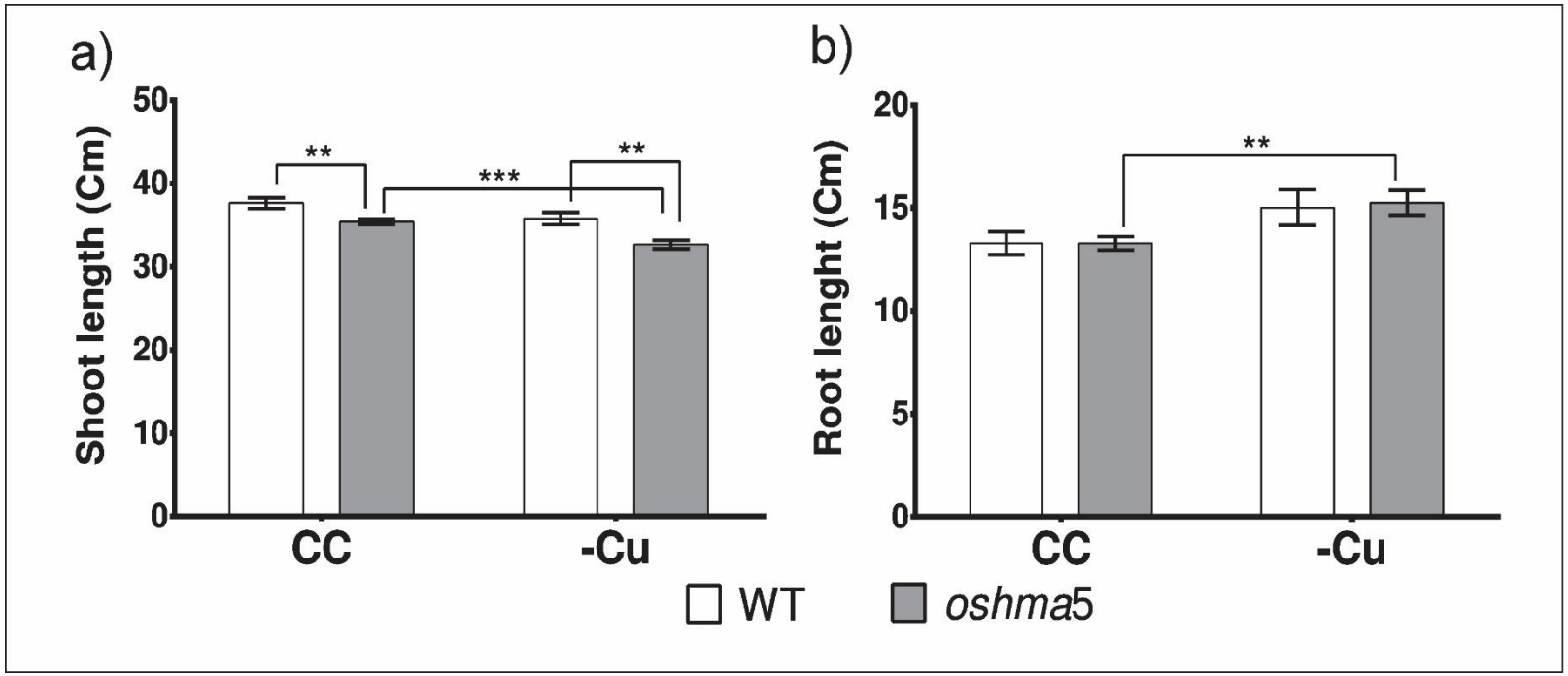

Figure 1. Shoot (a) and root (b) length in cm of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

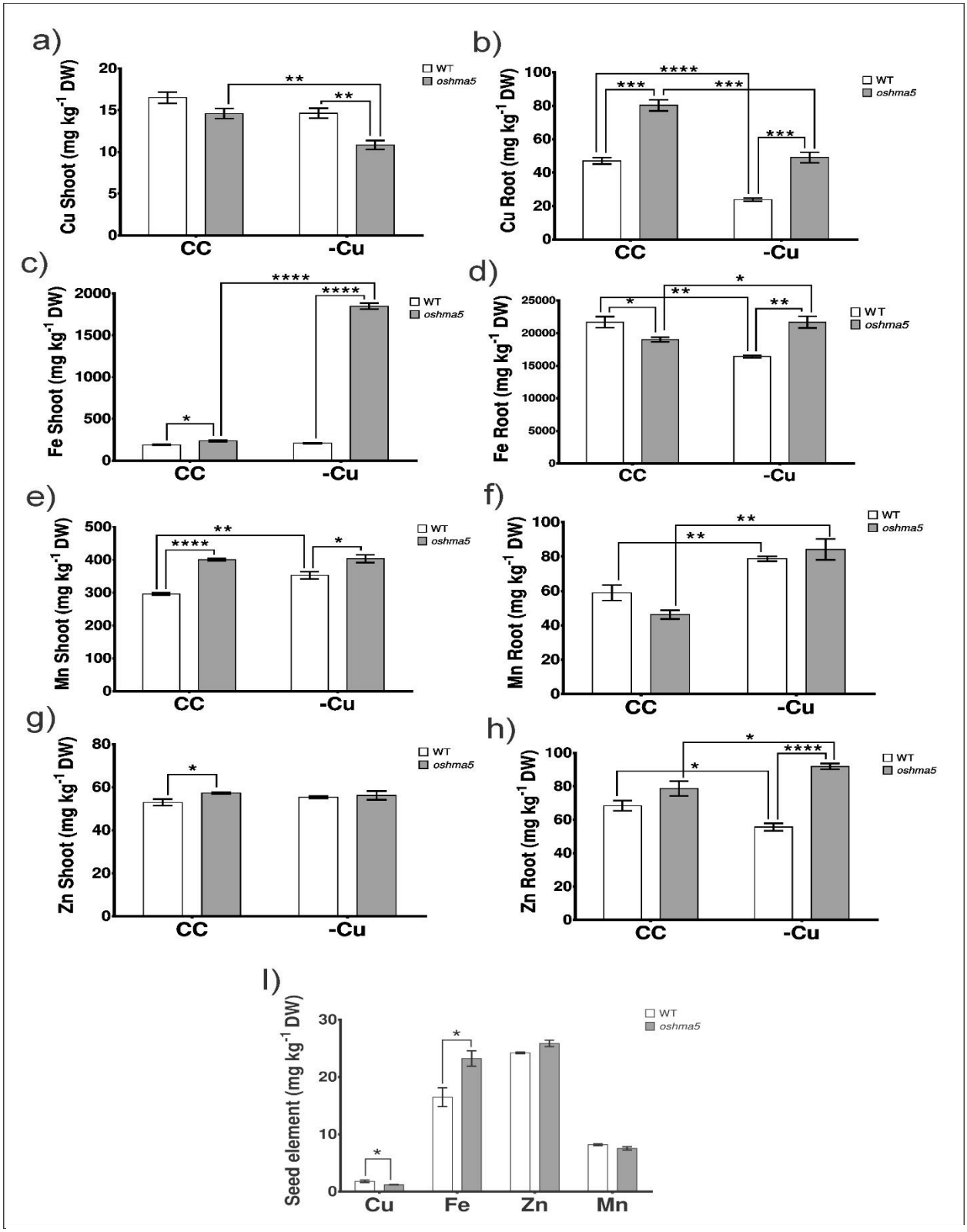

Figure 2. Concentration of Shoot copper (Cu) (a) root copper (b), shoot iron (Fe) (c), root iron (d), shoot zinc (Zn) (e), root zinc (f), shoot manganese (Mn) (g), root manganese (h) and seeds (i) expressed in mg kg^{-1} dry weight of rice (*Oryza sativa*) plants.

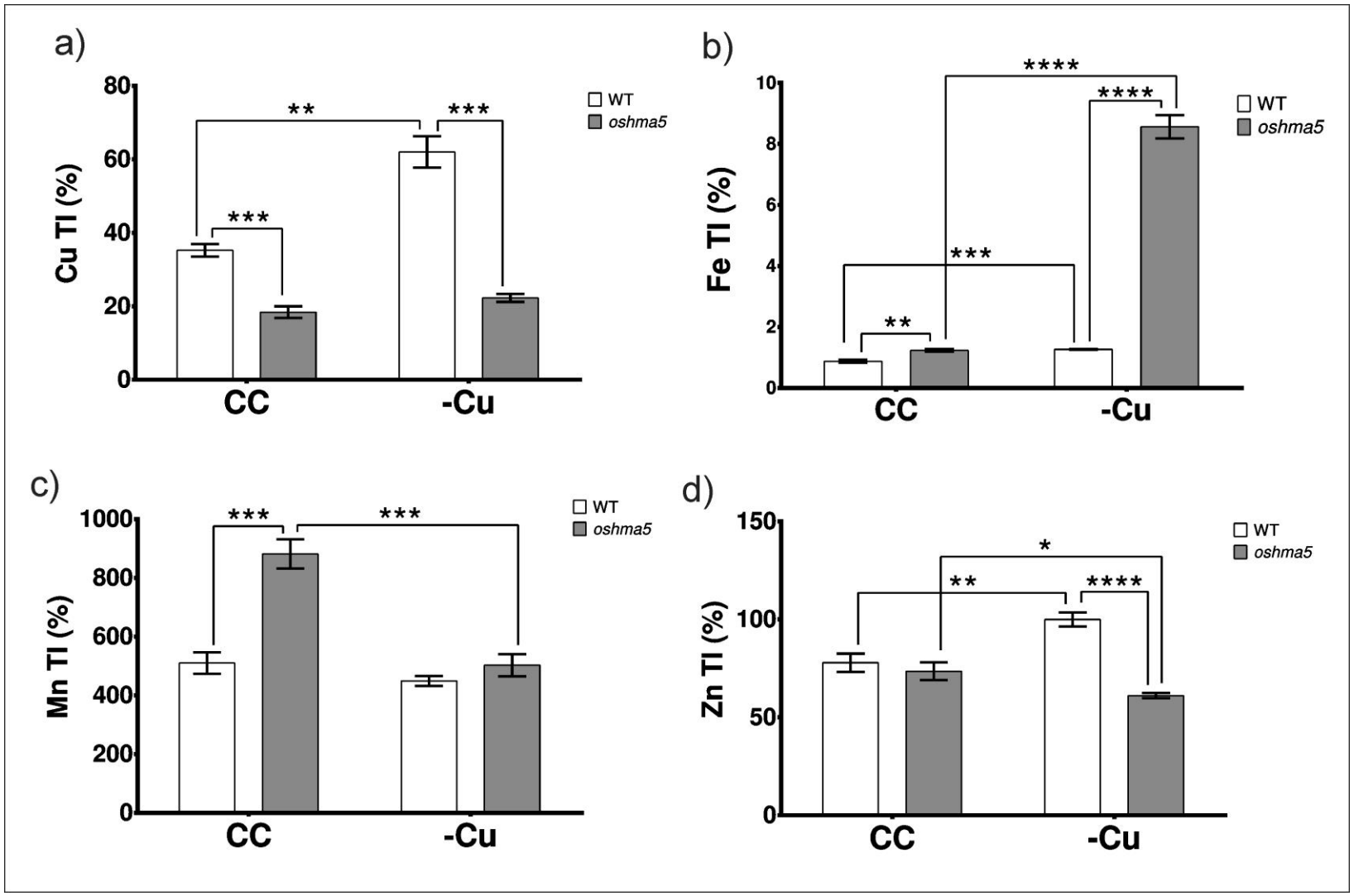

Figure 3. Element translocation index (TI%) f rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

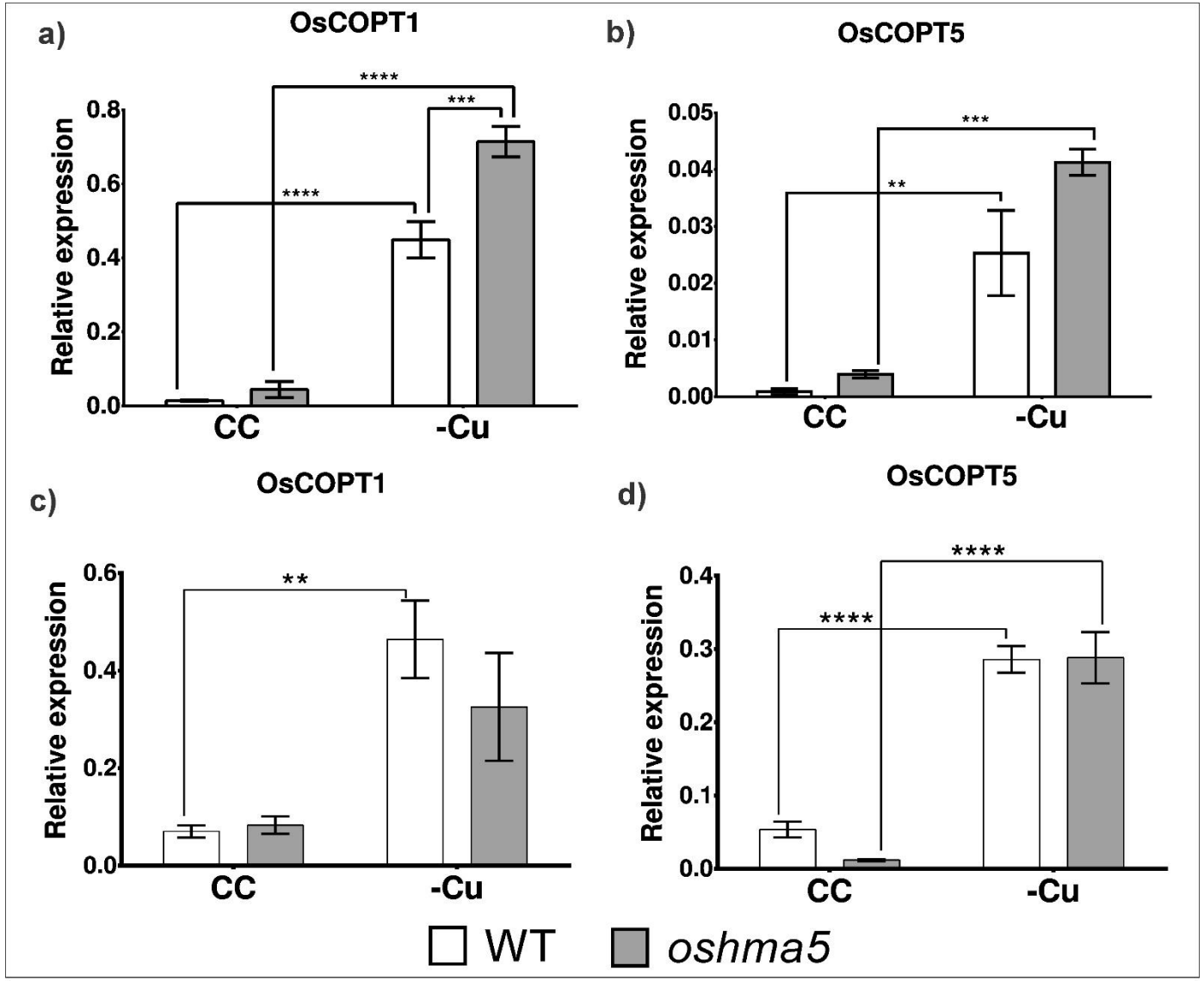

Figure 4. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of OsCOPT1 from shoot (a) and root (c) and OsCOPT5 from shoot (b) and root (c) genes expression of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

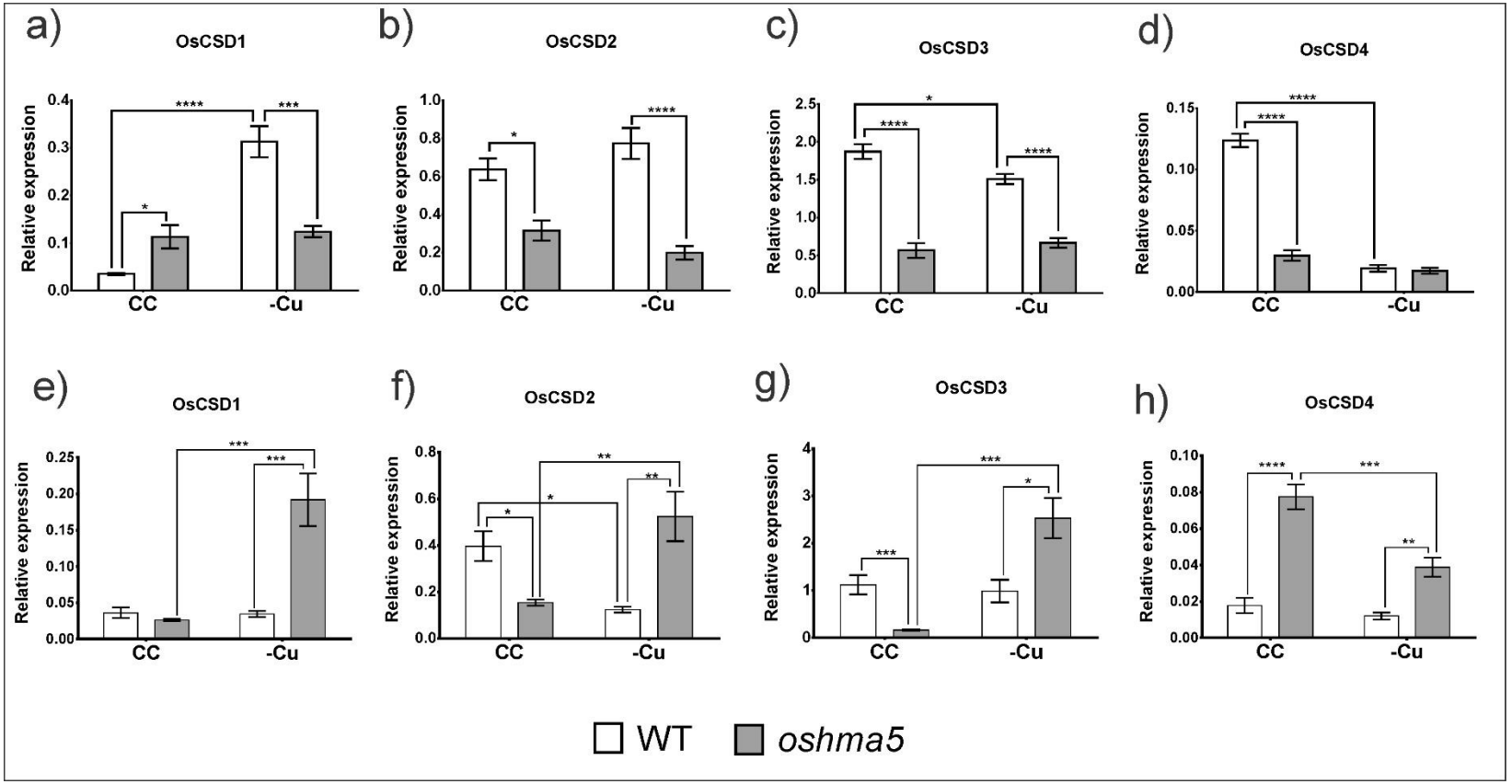

Figure 5. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of Cu-Zn superoxide dismutase; CSD1 from shoot (a) and root (e), CSD2 from shoot (b) and root (f), CSD3 from shoot (c) and root (g), CSD4 from shoot (d) and root (h) genes expression of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

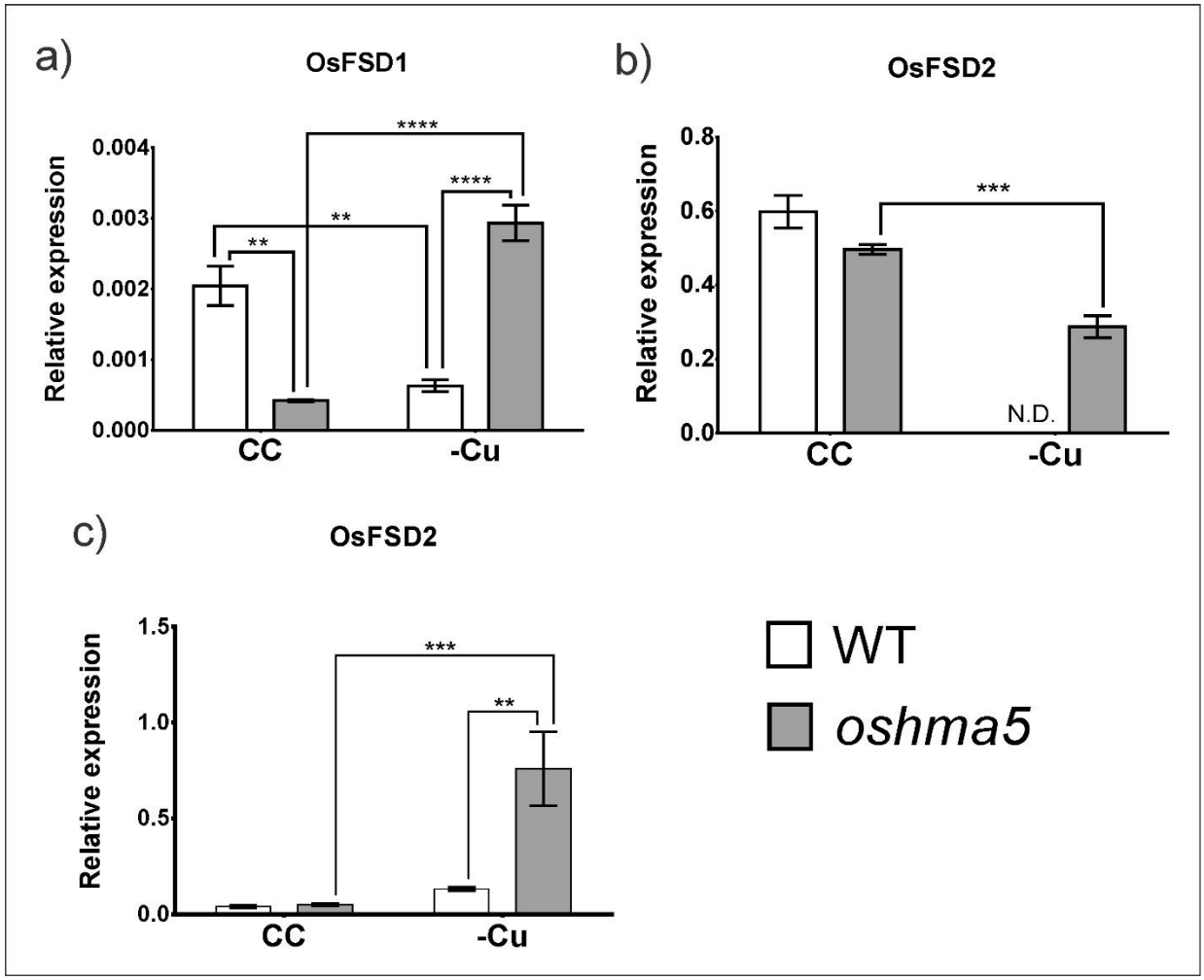

Figure 6. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of Iron superoxide dismutase; FSD1 from shoot (a) and root was not dectable, FSD2 from shoot (b) and root (c) genes expression of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

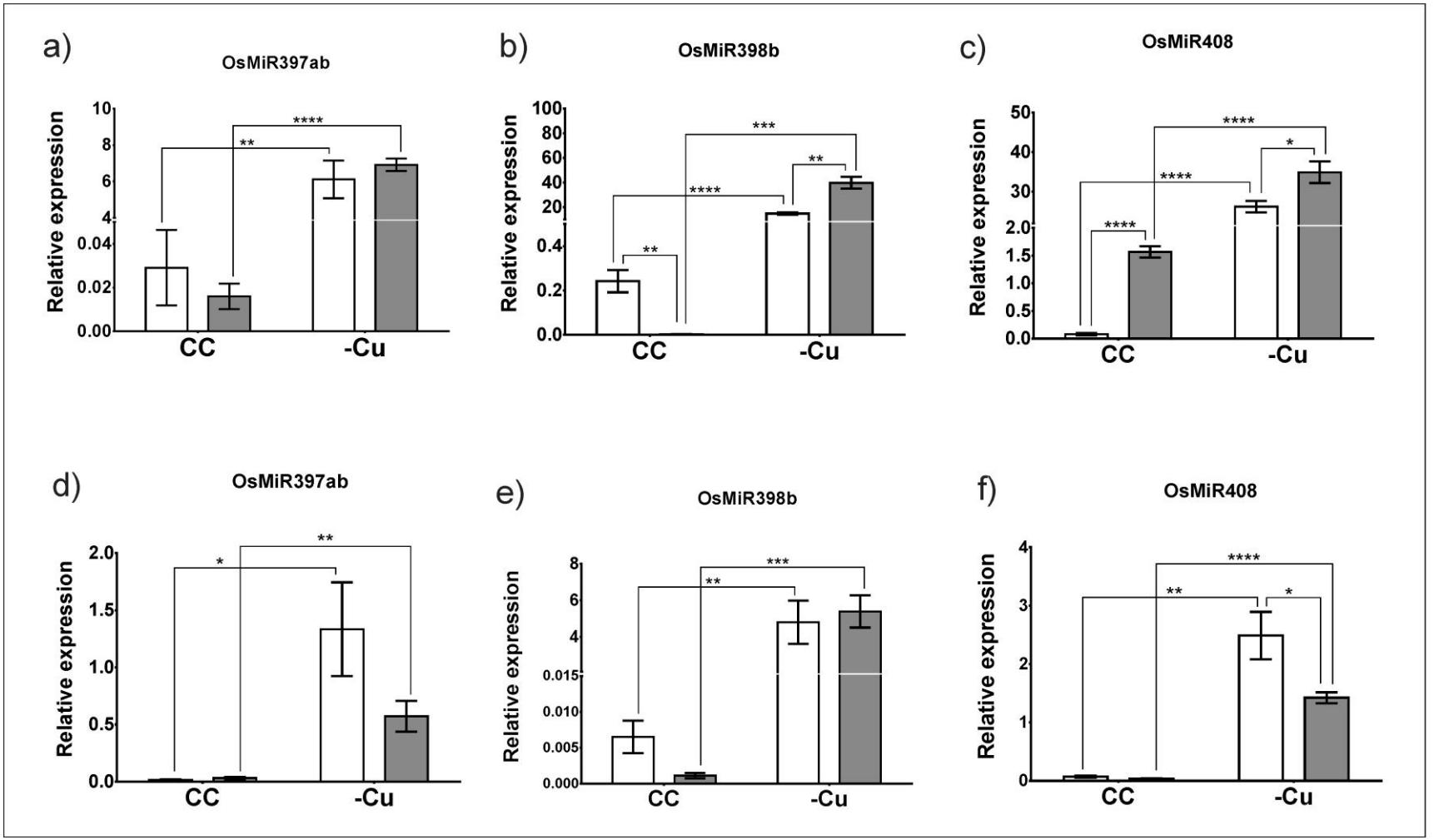

Figure 7. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of MiRNA397ab shoots (a) and roots (d), MiRNA398ab shoots (b) and roots (e), MiRNA408 shoots (c) and roots (f) genes expression of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

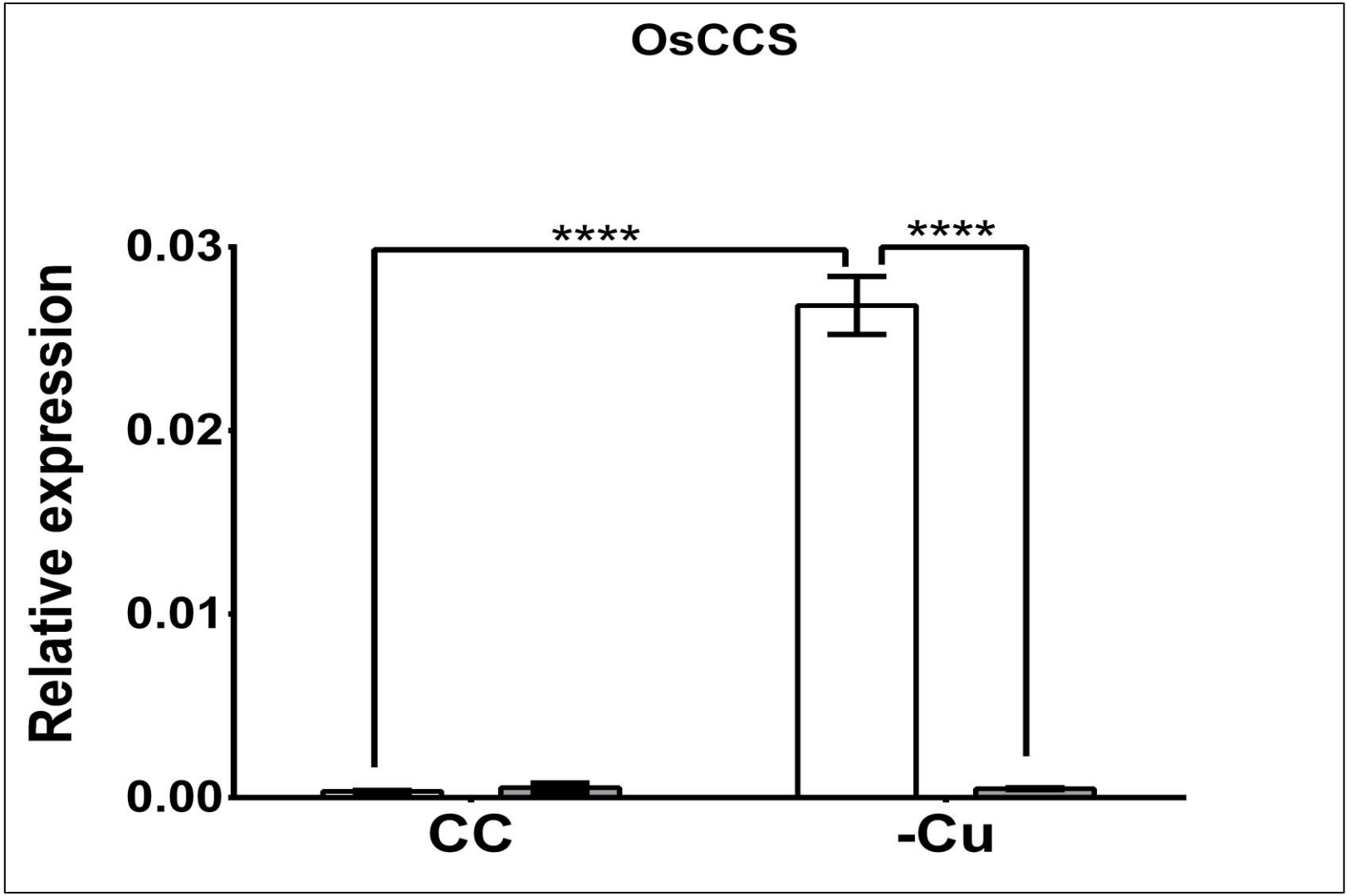

Figure 8. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of OsCCS from shoots genes expression of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).

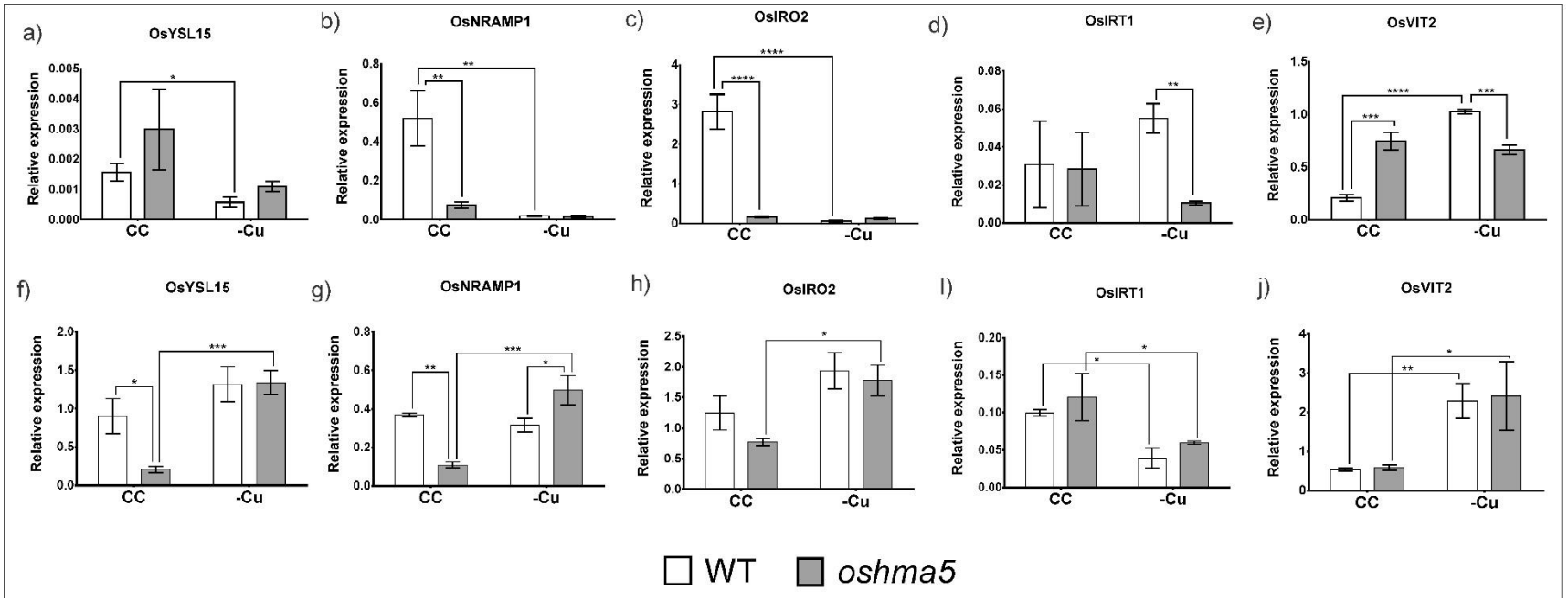

Figure 9. Reverse transcription quantitative real-time PCR analysis (RT-qPCR) of YSL15 shoots (a) and roots (f), NRAMP1 shoots (b) and roots (g), IRO2 shoots (c) and roots (h), IRT1 shoots d) and roots (i), VIT2 shoots (e) and roots (j) of rice (*Oryza sativa*) Nipponbare (WT) and mutant (*oshma5*).