論文の内容の要旨

応用生命化学専攻
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論文題目

**Elucidation of genes involved in cadmium distribution in rice through genome-wide association study and their characterization**

(イネのカドミウム蓄積に影響を及ぼす遺伝子のゲノムワイド関連解析を通じた同定とその特徴解明)

## Introduction

Food safety always receives worldwide attention. Cadmium (Cd), a toxic heavy metal, threatens human health mainly through accumulation in food and feed crops. Cd accumulation in vegetables and rice is one of the primary sources of human exposure to Cd. When Cd accumulates in the human body, many organs can be damaged by Cd: respiratory system, causing pneumonitis and destruction of the mucous membrane; kidney, causing proteinuria, kidney stones, glomerular and tubular damage; reproductive system, causing testicular necrosis and estrogen-like effects; skeletal system, causing loss of bone density, mineralization and Itai-itai diseases. Many other diseases can also be caused by Cd, such as anemia, heart failure, cancer, hypertension, and cerebral infarction. Thus, diminishing the accumulation of Cd in food is very important to protect humans from the threat of Cd.

Rice is an important staple crop in Asian countries. Elucidation of Cd accumulation mechanisms in rice is tightly related to human health, especially in Asian countries. Although several genes responsible for Cd uptake, transport, and detoxification, have been identified, the genetic and molecular mechanisms underlying Cd translocation in rice have not been fully understood.

With the genome-wide association study (GWAS), two candidate genes related to Cd accumulation in brown rice have been found in previous work in the laboratory of plant nutrition and fertilizers of the University of Tokyo. In this study, I performed an in-depth study on these two candidate genes (*OsMCR1*, *MATE Cd Reallocartion 1*, and *OsHIPP20*, *Heavy Metal-associated Isoprenylated Plant Proteins 20*). and analyzed their effect on Cd accumulation in brown rice and other portions of rice plants. Based on my finding, I elucidated the possible roles of newly identified genes in Cd transport and allocation in rice. I believe that my study helps further understanding of the process of Cd accumulation in rice and provides useful information for safety crop, a low Cd rice.

**Chapter I: Elucidation of the function of *OsMCR1* on Cd allocation in rice plants**

*OsMCR1* belongs to the *MATE* family. The genes of this family have been found to be involved in ions homeostasis or detoxifying toxic element in plants, such as Al, Fe, As and Cd. It has been known that MATE protein can work as an antiporter, which exclude organic ions using proton as a driving force.

**OsMCR1 affected Cd distribution in rice plants**

To observe the function of *OsMCR1* on Cd transport in rice plants, four independent CRISPR lines (*osmcr1-1*, *osmcr1-2*, *osmcr1-3* and *osmcr1-4*) of Nipponbare (NB) background were used. *osmcr1-1* and *osmcr1-2* were generated in previous work in the laboratory. *osmcr1-1* has a mutation at 5'UTR of *OsMCR1*, and the RNA accumulation of *OsMCR1* was decreased. *osmcr1-2* has a mutation at first exon on the coding region (CDS) of *OsMCR1*. *osmcr1-3* and *osmcr1-4* were generated by myself, and both of them have a mutation at first exon on the CDS of *OsMCR1*. To analyze the Cd distribution in rice, I cultured the mutants and NB in two types of soil: one is a commercial soil with a low level of Cd, and the other is obtained from Dr. Rai from Akita-Prefectural University with a high level of Cd. When grown in both types of soil, the Cd concentration in brown rice of four mutants was lower than NB. However, there was no difference between the mutant and the wild type in Cd concentration in the panicle node and the first node below the panicle node in both types of soil. The RNA accumulation of *OsMCR1* was examined by qRT-PCR, and the results showed that the RNA accumulation of *OsMCR1* in the flag leaf and the first node was low but high in panicle and tissues lower than the first node. These results indicated that *OsMCR1* affects Cd distribution in rice plants and mainly through Cd reallocation from lower portions of rice plants to seeds. I also treated the seedlings of *osmcr1-1*, *osmcr1-2* and NB with 1 μM Cd for one day or one week. The Cd concentration in roots and young leaf sheath of both mutants was decreased compared to NB. When treated with Cd for one week, the Cd concentration is much higher in the young leaf blade of both mutants but not in roots and young leaf sheat. These results indicated that *OsMCR1* probably affected the Cd uptake and reallocation in seedlings.

**OsMCR1 affected Zn and Mo concentration in brown rice**

Interestingly, in contrast to Cd, the concentration of Zn and Mo was increased in brown rice of mutants grown in the two types of soil compared to that of NB. It is likely that OsMCR1, as an antiporter, can exclude Zn or Mo out of the cell when Cd is transported into the cell. What is more, there was also a strong positive linear correlation between Zn and Mo concentration in tissues of plants, indicating that Zn and Mo were probably involved in some pathway together, and this pathway included OsMCR1. However, further experiments are needed to explain these results.

**Heterogeneous expression of OsMCR1 in Arabidopsis and yeast**

To confirm the function of *OsMCR1* on Cd transport, I introduced CDS of *OsMCR1* driven by CaMV 35S RNA promoter into Columbia-0 (Col-0), an ecotype of *Arabidopsis thaliana*. The root length was shorter in *OsMCR1* expression lines compared to Col-0 in the presence of Cd, while similar under normal condition. Further, Cd sensitivity was positively correlated with *OsMCR1* RNA accumulation level. Further, I expressed OsMCR1 in yeast, which has higher Cd than vector control. These results suggest that *OsMCR1* is able to transport Cd.

**OsMCR1 was preferentially localized to plasma membrane**

To identify the subcellular localization, N- or C-terminu GFP fusion protein of OsMCR1 was transiently expressed in rice protoplast. OsMCR1 was preferentially localized on the plasma membrane. However, the GFP signal can also be observed in other subcellular compartments: GFP-OsMCR1 is likely to be localized to the vacuole; OsMCR1-GFP was observed in a dot like structure.

Taken together, OsMCR1 have a Cd transport activity and affected Cd distribution in rice plants. It probably play a role on load Cd to the phloem and mediate Cd transport into brown rice.

**Chapter II: OsHIPP20 involved in Cd accumulation in rice plants**

OsHIPP20 is another candidate gene for Cd accumulation in brown rice. It belongs to the HIPPs family, which has a heavy mental associated domain (HMA) and a C-terminal isoprenylation motif. HMA has the ability to bind heavy metals like Cu, Zn and Cd. Isoprenylation is a post-translational protein modification essential for the interaction of the protein with other proteins or membranes. The genes that contain both the HMA domain and isoprenylation motif have been only found in plants. HIPPs are functioning in many ways: heavy metal homeostasis and detoxification (especially Cd); transcriptional regulator for the response to cold, salt and drought; plant-pathogen interactions.

**OsHIPP20 affected the Cd accumulation in rice plants**

To observe the function of OsHIPP20 in Cd accumulation in rice seeds, two CRISPR lines (*oshipp20-1* and *oshipp20-2*) in the cultivar Akebono , In addition, *oshipp20-3*, a *Tos17* insertion line were used. Both *oshipp20-1* and *oshipp20-2* have a mutation at the second exon of *OsHIPP20*. *oshipp20-3* has a *Tos17* insertion at the 3'UTR, whose RNA accumulation was decreased by fifty-fold. The Cd concentration of brown rice in the mutants was decreased when grown in the high Cd soil compared to wild type (WT). However, there was no difference under low Cd soil. (I could not understand your sentence here as there are two consective "However")These results indicated that *OsHIPP20* was involved in Cd transport to brown rice under high Cd condition. The Cd concentration was also changed in some tissues below panicle in mutants grown in the low level of Cd and high level of Cd. These results indicate that OsHIPP20 affected Cd accumulation in rice plants.

I also examined the Cd concentration in two-week-old seedlings of *oshipp20-3* and NB. When treated with 1 μM Cd for one day, the Cd concentration in young leaves was lower in *oshipp20-3* compared to NB. There was an increased Cd concentration in old leaves after one week of Cd treatment. These results suggested that OsHIPP20 also affected Cd accumulation in rice seedlings.

**OsHIPP20 and GFP fusion protein was observed nucleaus and other**

To confirm the subcellular localization of OsHIPP20, I generated fusion protein with GFP at the N-terminus and C-terminus of OsHIPP20. The GFP signal was observed in the nucleus when GFP was fused at the N-terminus of OsHIPP20. When fused to C-terminus, GFP signal was observed as large dot,

which is not nucleus. The C-terminus of HIPPs contains an isoprenylation motif, which may be required for the protein-protein interaction or membrane anchoring. It is possible that GFP fused at C-terminus prevent a protein interaction and leads to the abnormal localization.

**OsHIPP20 was toxic to *Arabidopsis* and yeast**

To further confirm the function of *OsHIPP20* on Cd accumulation. I introduced CDS of *OsHIPP20* driven by CaMV 35S RNA promoter into promoter into Col-0 of *A. thaliana*, and CDS of *OsHIPP20* driven by GAPDH promoter or GAL1 promoter in yeast strain W303. To my surprise, it seems that OsHIPP20 is toxic to *A. thaliana* and yeast. Heterogeneous expression of *OsHIPP20* severely inhibited the growth of both *Arabidopsis* and yeast. So, it is hard to observe the effect of *OsHIPP20* on Cd accumulation in other species.

Taken together, OsHIPP20 can affect the Cd accumulation in plants. OsHIPP20 probably interact with transcription factor in nucleus to regulate other genes expression.

**Conclusion**

In this thesis, I confirmed the involvement of two genes in Cd accumulation in brown rice: *OsMCR1* and *OsHIPP20*. Knockout of either gene can significantly decrease the Cd concentration in brown rice. *OsMCR1* was involved in Cd reallocation from old tissues to panicle. It may load the Cd into the phloem in old tissues for translocation. However, the tissue localization of OsMCR1 is needed to confirm this hypothesis. OsHIPP20 also affected the Cd accumulation in tissues below the panicle and was related to transport Cd into brown rice instead of husk. OsHIPP20 probably interacts with transcription factors to regulate the gene that is related to Cd transport. However, how OsHIPP20 regulates the Cd accumulation in rice plants is still unknown. Much more work is needed to find the interaction proteins and downstream genes to elucidate the intrinsic mechanisms.