博士論文（要約）

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)

肖 華

XIAO HUA

Abstract

Food safety always receives worldwide attention. However, cadmium (Cd), a toxic heavy metal, threatens human health mainly through food accumulation and feed crops. Cd accumulation in vegetables and rice is one of the primary sources of human exposure to Cd. Rice is an important staple crop in Asian countries. Thus, elucidation of how Cd accumulated in rice is tightly related to human health, especially in Asian countries. Although some genes responsible for Cd uptake, transport and detoxification have been identified, the genetic and molecular mechanisms underlying metal homeostasis and rice translocation have not been fully understood.

With the help of a powerful tool – genome-wide association study (GWAS), two candidate genes related to Cd accumulation in brown rice have been found in previous work in the lab 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* and *OsHIPP20*) identified by GWAS and analyzed their effect on Cd accumulation in grain and other portions of rice plants. Based on my findings I elucidated possible roles of newly identified genes in Cd transport and allocation in rice. I believe that the present study will help further understand the process of Cd accumulation in rice and provide useful information for crop improvement.

In chapter I, I confirmed the function of *OsMCR1* and tried to elucidate how it affected the Cd accumulation plants.

When grown in both kinds of soil, the Cd concentration in brown rice of four mutants was decreased compared to NB. The Cd concentration in some other tissues was also affected in mutants grown in high level of Cd soil. However, there was no difference observed in tissues between the panicle node and the first node below the panicle node in all mutants grown in both soil types. The RNA accumulation of *OsMCR1* was checked by qRT-PCR and the results showed that the RNA accumulation of *OsMCR1* in the flag leaf and the first node was very low but very high in panicle and tissues lower than the first node. These results indicated that *OsMCR1* affect Cd distribution in rice plants and mainly reallocate Cd 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 1 day or 1 week. The Cd concentration in toots and young leaf sheath of the 2-week-old seedling of both mutants was decreased compared to NB when treated with Cd for 1 day. The Cd concentration is much higher in the young leaf blade of both mutants when treated

with Cd for 1 week. These results indicated that *OsMCR1* probably affected the Cd uptake in root in a short time Cd stress but affected Cd remobilization in young leaves in the long term.

Four CRISP lines (*osmcr1-1*, *1-2*, *1-3* and *1-4*) of Nipponbare (NB) background were used to confirm the function of OsMCR1 on Cd transport in rice plants. *osmcr1-1* and *osmcr1-2* were generated in previous work in the lab of plant nutrition and fertilizers of the university of Tokyo. *osmcr1-1* has a mutation at 5'UTR of *OsMCR1*, and the RNA accumulation of *OsMCR1* was decreased in 42 day after flower (DAF) seeds of *osmcr1-2* compared to that of NB when checked by qRT-PCR. *osmcr1-1* has a mutation at first exon on the coding region (CDS) of *OsMCR1*. *osmcr1-3* and *osmcr1-4* were generated by me and both have a mutation at first exon on the CDS of *OsMCR1*. I cultured the mutants with NB in two kinds 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 high level of Cd. the Cd concentration in brown rice of mutants was decreased compared to NB. The Cd concentration in some other tissues was also affected in mutants, indicating that OsMCR1 affects Cd distribution in rice plants. Interestingly, in contrast to Cd, the concentration of Zn and Mo was increased in brown rice of mutants grown in both kinds 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 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 probably involved in some pathway together and this pathway included OsMCR1. However, further experiments are needed to explain these results.

To further confirm function of *OsMCR1* on Cd transport. I introduced CDS of *OsMCR1* driven by CaMV 35S promoter into Columbia-0 (Col-0) an ecotype of *Arabidopsis thaliana*, and CDS of OsMCR1 driven by GAPDH promoter in yeast strain W303. Heterogeneous expression of *OsMCR1* results in an increase sensitivity to Cd in *Arabidopsis*. Under normal MGRL culture medium, there was no difference between Col-0 and *OsMCR1* expression lines. However, the growth of root was inhibited in *OsMCR1* expression lines compared to Col-0 when Cd (10 μM or 25 μM) was added. The Cd sensitive level was also related to the *OsMCR1* RNA accumulation level in *OsMCR1* expression lines, the higher of the RNA accumulation the stronger inhibition on root growth. In the yeast, the Cd concentration was increased when the yeast expressed *OsMCR1* compared to the yeast transformed with empty vector. All these results indicated that *OsMCR1* is related to Cd transport.

The fusion protein of OsMCR1 with GFP ligated to N-terminus or C-terminus was generated. The localization of OsMCR1 was checked by observing the GFP signal in rice protoplast under confocal microscope.

It seems that OsMCR1 is preferentially located on the plasma membrane. However, the GFP signal can also be observed in subcellular compartments likely to be the vacuole when GFP is fused to N-terminus of OsMCR1 or forming aggregates when GFP fused C-terminus of OsMCR1. However, whether this property of OsMCR1 is related to Cd transport or detoxification is still unknown.

In chapter II: I confirmed the effect of another gene *OsHIPP20* on Cd accumulation in rice plants

OsHIPP20 belongs to the HIPPs family that have a heavy metal 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 process and essential for the interaction of the protein with other proteins or membranes. Genes that contain both the HMA domain and isoprenylation motif were only found in plants.

To confirm with OsHIPP20 affected Cd accumulation in rice seeds. Two CRISPR lines (*oshipp20-1* and *oshipp20-2*) in the cultivar Akebono, an ecotype of *Oryza sativa* used for previous GWAS study, background, and *oshipp20-3*, a *TOS17* line (*oshipp20-3*) were used. *oshipp20-1* and *oshipp20-2* has a mutation at the second exon of *OsHIPP20*. *oshipp20-3* has a *TOS17* insertion at the 3'UTR and the *OsHIPP20* RNA accumulation was decreased in germinated seeds when checked by qRT-PCR. The Cd concentration in mutants tested was decreased when cultured in high level of Cd soil compared to wild type (WT). However, I did not observe difference of Cd concentration in panicle (brown rice, husk, rachis and panicle node) between *oshipp20-1*, *oshipp20-2*, *oshipp20-3* and their WT (Akebono or NB) when grown in low level of Cd soil. However, there was increased Cd concentration in husk in mutants when grown in high level of Cd soil, and decreased Cd concentration in brown rice, rachis, and panicle node. This result indicated that *OsHIPP20* was related to Cd transport into panicle and mediated Cd accumulation in brown rice instead of husk. Because there are many other differences between the two kinds of soil. The Cd concentration was decreased in some tissues below panicle in mutants grown in low level of Cd and high level of Cd, except flag leaf blade that Cd concentration increased in mutants when grown high Cd soil. These results indicated that OsHIPP20 affected the Cd accumulation in rice plants. However, it is possible but hard to say that the different Cd level in soil is the reason for having difference in panicle between mutants and WT grown in high level of Cd but not in low level of Cd.

I also checked the Cd concentration in 2-week-old seedlings of *oshipp20-3* and NB. When treated with 1 μM Cd for 1 day, the Cd concentration in young leaves was decreased in *oshipp20-3*. And there was an

increased Cd concentration in old leaves after 1 week Cd treatment. These results suggested that OsHIPP20 also affected Cd accumulation in rice seedlings.

To confirm the subcellular localization of OsHIPP20, I generated fusion protein with GFP at N-terminus and C-terminus of OsHIPP20. The GFP signal was observed in nucleus when GFP fused at the N-terminus of OsMCR1. However, The GFP signal was aggregated together other than nucleus when fused to C-terminus. The C-terminus of *HIPPs* contains isoprenylation motif that is involves addition of a C–terminal hydrophobic anchor and important for the interaction with the protein or membranes. It is possible that GFP fused at C-terminus lead to an abnormal localization of OsHIPP20. Thus, OsHIPP20 is likely a nucleus located protein. where Subcellular location in protoplast showed that OsHIPP20 should be located at the nucleus. OsHIPP20 probably interact with transcription factor and regulate genes that related to Cd transportation.

To further confirm function of OsHIPP20 on Cd accumulation. I introduced CDS of *OsHIPP20* driven by CaMV 35S promoter into Col-0 of *Arabidopsis 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 *Arabidopsis* 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.