論文の内容の要旨

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論文題目 Investigation on the role of gene regulation between chlorophyll biosynthesis and Fe accumulation in rice

（イネのクロロフィル合成と鉄集積に関わる遺伝子発現制御に関する研究）

Rice is the global staple food and feeds over half of the world's population. Over 90 percent of the world's rice is produced and consumed in Asia. Improving the yield of rice can eradicate poverty and hunger problems, however rice yield is influenced by micronutrient deficiency. Among micronutrient deficiencies, iron (Fe) deficiency is one of most serious problems affecting rice productivity and quality. Thus, we can improve plant product quality through increasing Fe content by Fe biofortification. In this context, characterization of the molecular and physiological mechanisms of Fe metabolism in plants can impel in agriculture and biotechnology to fit the goal of sustainable production of more nutrient food.

In rice, Fe is the most common constituent for several enzymes and pigments, and Fe is essential for chlorophyll biosynthesis, Fe-S cluster assembly, photosynthesis and energy transformation that occur in chloroplasts. Therefore, characterization of Fe deficiency inducible genes will help us further understand the mechanism between Fe accumulation and chlorophyll biosynthesis throughout the plants' lives, and is important for developing Fe deficient tolerance plants to improve quality and quantity of crops in calcareous soils.

In the thesis, one novel Fe deficiency induced protein kinase domain containing protein gene was identified and its localization and functions involved in Fe deficiency response and plants growth were investigated. To clarify the molecular mechanism that regulates Fe metabolism in graminaceous plants, 40 thousand genes in rice were characterized by microarray analysis under Fe deficiency stress. Among Fe-deficiency inducible genes, around 50 genes, whose expression pattern was similar to that of mugineic acid related genes, were selected. Of these genes, one gene encoding protein kinase domain containing protein (PK protein) was strongly expressed in roots and young leaves during Fe deficiency stress. To investigate functions of PK protein in rice, one *pk-tos* mutant was identified and, in that mutant, one retrotransposon tos17, which obstructed gene expression was inserted into *PK* gene. *pk-tos* mutants

showed chlorotic leave phenotype, which was similar to Fe deficiency phenotype, however they contained significant high amounts of Fe. Expression analysis of Fe homeostasis genes (*Osferritins* and *OsIRO2*) was carried out in *pk-tos* mutants. In *pk-tos1-1* mutant, expression of *Osferritins* was increased under control and Fe deficient condition in shoots. The expression of *OsIRO2* in *pk-tos1-1* was reduced in both shoots and roots under Fe deficiency conditions as well as under control conditions in compared with that in WT. The SPAD value of *pk-tos* mutants was almost half of WT, meaning that chlorophyll contents in *pk-tos* mutants were lower than those in WT. Chlorophyll contents were analyzed to elucidate the reason of cholortic phenotype of *pk-tos* mutants. For WT, the ratio of chlorophyll a (chl a) and chlorophyll b (chl b) was all around 3:1 under Fe sufficient and deficient conditions. For *pk-tos* mutant, chlorophyll a (chl a) contents decreased a lot compared with those of WT, and there was no chl b in *pk-tos* mutants. *PK* expression was upregulated in roots and young leaves but repressed in old leaves in early stage of Fe deficiency, showing that *PK* expressing tissues require more Fe. High Fe concentration in *pk-tos* mutants reflect that Fe deficiency is not the reason of clorotic phenotype. Furthermore, high *Osferritins* expression, low *OsIRO2* induction showed high amounts of stored Fe and low sensitivity to Fe deficiency in *pk-tos* mutants. Content of chl a almost reduced by a third and chl b decreased to zero in *pk-tos* mutants in comparing with WT, indicating that chl a biosynthesis pathway could be conducted in *pk-tos* mutants and chl b production processes was blocked due to inhibition of *PK* expression. These results suggest that PK might have relation with chlorophyll biosynthesis-related proteins, and specifically to chlorophyllide a oxidase.

Besides chlorophyll biosynthesis, Fe also plays important part in photosynthesis and electron transfer. Fe requirement of photosynthesis and chlorophyll biosynthesis which happen in chloroplasts make them become the Fe richest organelle in plant cell that contain 80-90% of Fe in leaf cell. However, excess Fe generate reactive oxygen species (ROS) which cause oxidative damage for plants. In chloroplast, free Fe from reduced Fe-S clusters and ROS like H_2O_2 produced by the photosynthetic chain are close proximity to each other causing formation of hydroxyl radicals via Fenton reaction. Thus, it is important to keep the homeostasis between free Fe and ROS via the fine regulation of Fe related genes involved in chloroplast Fe storage, transport. In the thesis, *OsIRO3* was found and it might involve in the maintenance of Fe balance between cytoplasm and vacuole/or plastid under Fe deficiency through the regulation of Fe related gene regulation.

OsIRO3 was reported as a first repressor related to Fe-homoeostasis gene regulation by the evidence with overexpression rice. Phylogenetic analysis showed that *OsIRO3* is the closest orthologue to *PYPEYE* in Arabidopsis. Both proteins have a role in negative regulation of gene expression. While for *OsIRO3*, only overexpression line has been reported so that knock down/out of *OsIRO3* will help us further understand and compare Fe deficiency mechanism in rice and Arabidopsis. Experiments using Os*IRO3* repressed rice by both RNA interference method and T-DNA insertion showed that *OsIRO3* knockdown mutants showed brown spots on the leaves and became damaged after 8 days of Fe deficient treatment. Moreover, *OsIRO3* knockout rice

has been developed using CRISPR/Cas9 system and phenotype of *OsIRO3* knockout mutants was more severe than *OsIRO3* knockdown rice. The mutants showed strong lesion on young leaves after 3-4 days' Fe deficiency treatment, causing the death of younger leaves within 1 or 2 days. The knock down/out mutants all displayed Fe toxic phenotype on younger leaves under Fe-deficiency treatment with significant amounts of *Osferritins* genes. Fe concentration was significantly higher in the younger leaves of *OsIRO3* knockout mutants. In addition, significantly induced *OsNAC4* expression and H_2O_2 amounts in the youngest leaves of *OsIRO3* knock down/out plants by Fe deficiency indicated that *OsIRO3* knock down/out mutants displayed the cell death in younger leaves due to the Fe-mediated production of ROS leading to oxidative stress under Fe deficiency. On the other hand, Fe efflux and influx of plastid as well as Fe storage are of importance to maintain Fe homeostasis. Furthermore, Fe uptake transporter genes like *OsNRAMP1* are upregulated and Fe efflux transporter genes like *OsVIT1*, *OsVIT2* and *OsNRAMP6* are downregulated under Fe deficiency. *OsIRO3* upregulated by Fe deficiency possibly represses or induces the expression of Fe influx transporter genes and Fe influx transporter genes to possess an equal homeostasis in cell.

Gene expression in rice roots and shoots under Fe deficiency treatment was investigated by microarray. Large number of genes were upregulated by Fe deficiency in rice. Some of them have been already well characterized and play important roles in Fe acquisition and homeostasis. Fe^{2+} transporter gene *OsIRT1* and DMA synthesis genes (*OsNAS1*, *OsNAS2*, *OsTOM1*) were all upregulated by Fe deficiency and showed important roles in regulation of Fe metabolism. Thus, 8 novel genes which were activated by Fe deficiency including *PK* gene were selected and constructs for knockout mutants using CRISPR/Cas9 system were designed. Then, rice transformation and homozygous lines screening were conducted. 3 lines of homozygous for *PK*, 1 homozygous line of Zinc finger, RING/FYVE/PHD-type domain containing protein (*OsRFP*) was screened successfully in T1 generation. T2 generation of the homozygous line of *OsPFR* was obtained. Plant growth including shoot and root elongation was impeded by knockout of *OsPFR*. Cu concentration was significantly reduced in *OsPFR* knockout line. Thus, it is conjectured that *OsPFR* might play a role in regulating metal uptake and maintaining the normal growth of plants. Further researches need to be conducted to characterize function of *OsRFP* in detail by knockout plants.

This research represents that amounts of genes were upregulated by Fe deficiency. Among them, *PK* gene significantly upregulated by Fe deficiency might be involved in chlorophyll biosynthesis pathway. *OsIRO3* significantly upregulated by Fe deficiency might maintain Fe efflux/influx of chloroplast and buffer the balance of free Fe with ROS generated by photosynthesis through the regulation of expression of Fe related downstream genes. *OsPFR* might play a role in regulating metal uptake and maintaining the normal growth of plants. Therefore, strict control of Fe homeostasis is the key to guarantee proper plant growth and development. Further analysis will be conducted to identify and characterize other Fe deficiency induced genes through CRISPR/Cas9 system. Clarification of Fe deficiency responsible genes would give us more information on the mechanisms of Fe regulation between chlorophyll biosynthesis, photosynthesis and Fe accumulation.