論文の内容の要旨

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論文題目

**Molecular Mechanisms of Nutrient-Stress Adaptation in *Arabidopsis thaliana***
**（シロイヌナズナの栄養ストレス適応の分子機構）**

In this thesis I describe analyses on plant response and tolerance to nutrient deficiency in model plant *Arabidopsis thaliana*.

There are seventeen essential elements for plant growth and reproduction (Cakmak and Römheld 1997). Among them, nitrogen, phosphorus, and potassium are often added to fields as fertilizers to improve crop production. This means these major nutrients are deficient in many soils. Some other nutrients (minor nutrients) are also deficient in soils, and cause agricultural problems around the world. Diverse methods have been adopted by researchers to reveal genetic mechanisms for response and tolerance to nutrient deficiency. Straight-forward screening of deficiency-sensitive loss-of-function mutants was only successful in restricted numbers of studies, including identification of LKS1 which regulates a potassium transporter AKT1 (Xu et al. 2006). There are also reports on identification of nutrient transporters with the use of toxic analogs, for example identification of sulfate transporter SULTR1;2 from selenate-tolerant mutants (Shibagaki et al. 2002) and identification of silicon transporters OsLsi1 and OsLsi2 from germanium-tolerant mutants (Ma et al. 2002, Ma et al. 2006, Ma et al. 2007).

Induction of expression by nutrient deficiency is also a cue to identify genes. OsPTF1, a transcription factor which improves rice growth under phosphorus deficiency, was identified from its induction by phosphorus deficiency (Yi et al. 2005) and a boron transporter NIP5;1 was identified from its induction by boron deficiency (Takano et al. 2006). Several other methods were taken to identify various genes involved in the tolerance to nutrient deficiency (for only a small part of examples, Hirsch et al. 1998, Hamburger et al. 2002).

In this study, I aimed to reveal novel genetic mechanisms that improve plant tolerance to nutrient deficiency. As described above, there have been several strategies to take for this goal, and I took the same ways (chapters 1,3,4) or I also tried a novel method (chapters 5,6) in this study. In chapter 2, I established a novel high-throughput method to accelerate genetic studies. Although these extensive trials did not identify genes that improve plant tolerance to nutrient deficiency, I identified several novel aspects of plant response to nutrient deficiency. Most importantly, recent results indicate novel methods to improve plant tolerance to nutrient deficiency through modification of functionally characterized genes.

## Chapter 1. The *BIG* gene is involved in regulation of sulfur deficiency-responsive genes in *Arabidopsis thaliana*

*asr1* is one of the low-sulfur response mutants isolated by Dr. Naoko Ohkama-Ohtsu. Expression of *green fluorescence protein* gene driven by a low-sulfur responsive promoter is

upregulated in this mutant. After collaboratory map-based cloning, nonsense mutations were identified in the *BIG* gene locus in both two alleles. Low-sulfur response was also upregulated in other *big* alleles. To know the factor which upregulate low-sulfur response in *big* mutants, indole-3-acetic acid and an auxin transport inhibitor was applied to plants, because disturbed auxin transport is the most important effect know to be caused by *big* mutation. These two chemicals induced low-sulfur responses in *Arabidopsis*, although in some different pattern from those observed in *big* mutants. Thus we concluded that the *BIG* gene affects low-sulfur response independently of auxin metabolism.

**Chapter 2. A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis**

DNA extraction protocols from *Arabidopsis* required several steps, such as boiling, ethanol precipitation, or drying in vacuum. A number of simplified and rapid protocols of DNA extraction for *Arabidopsis* have been reported, however, they still required several steps. Here I established a novel one-step DNA extraction protocol for uses in polymerase chain reaction (PCR). Based on several rapid protocols, I tested several conditions. Among them, a buffer which is a dilution of a former extraction buffer (Edwards et al. 1991) successfully extracted DNA in one-step. The DNA solutions were successfully analyzed for detection of polymorphisms between *Arabidopsis* accessions or detection of T-DNA insertion (performed by Ms. Yoko Ide) by PCR. Other rapid one-step protocols are also reported by other research group (Berendzen et al. 2005). These protocols are suitable for saving time, labor, and cost of DNA extraction from *Arabidopsis*.

**Chapter 3. Identification of novel *Arabidopsis thaliana* genes which are induced by high levels of boron**

In tobacco BY-2 cultured cells, several genes induced by boron deficiency are reported (Kobayashi et al. 2004). In *Arabidopsis*, *NIP5;1* was the only gene which was known to be induced by boron deficiency. NIP5;1 is a boron channel which rescues plant growth under boron deficiency (Takano et al. 2006). To know the transcriptome-level regulation of gene expressions by boron deficiency and boron toxicity, transcriptome analysis was performed in *Arabidopsis*. Among ~12,000 genes detected in this analysis, *NIP5;1* was the only gene whose expression was induced only by boron deficiency more than 2.5-fold, indicating specific mechanism for regulation of *NIP5;1* gene expression by boron deficiency. In this analysis several genes whose expressions are induced only by boron toxicity were also identified. This is the first identification of high-boron induced genes.

**Chapter 4. Regulation of gene expression by boron deficiency around root tip of *Arabidopsis thaliana* and involvement of *WRKY6* in regulation**

To know the functions of high/low-boron responsive genes identified in chapter 3, mutants which possess T-DNA insertions in the exons of the induced genes were selected. These mutants were analyzed for tolerance to high/low-boron. Mutant designated as *wrky6-3* showed reproducible but not stable phenotype. Root elongation of *wrky6-3* was sometimes worse than wild-type under boron deficiency. Promoter activity of the *WRKY6* gene was constantly induced near the root tip under boron deficiency. *WRKY6* is a transcription factor. To know the effect of *WRKY6* on gene expressions under boron deficiency, another

transcriptome analysis was performed around root tip. Many genes were induced by boron deficiency around root tip after a long-term boron deficiency. Inductions of some of these genes were inhibited in *wrky6-3* mutant, showing involvement of *WRKY6* in regulation of gene expressions by boron deficiency.

### Chapter 5. Screening of *Arabidopsis thaliana* gain-of-function mutants under nutrient deficiency

Screening of loss-of-function mutants like ethylmethanesulfonate-mutagenized lines has been applied to screenings for genes functioning in tolerance to nutrient deficiency, although only restricted numbers of successful screenings are found (ex. Xu et al. 2006). This will be partially because naturally smaller mutants also appear in the screening and it is hard to distinguish sensitive individuals to nutrient deficiency from naturally smaller mutants. We should also be aware that a gene which functions for tolerance to nutrient deficiency does not necessarily improve growth when ectopically overexpressed. Screening of gain-of-function mutants may be an effective alternative. In this chapter, screening of gain-of-function mutants was performed under nutrient deficiency for the first time. After screening ~450,000 plants, although, no obviously tolerant line was identified. Because control genes such as *LKS1* (Xu et al. 2006) and *BOR1* (Miwa et al. 2006), which improve tolerance to potassium deficiency and boron deficiency, were also not recovered in the screening, the number of plants was not enough in the screening. Developments of high-throughput screening conditions are waited for for completion of the screening. In FOX lines, position effect will reduce screening efficiency. Introduction of sea urchin insulator sequence upstream of 35S promoter in FOX lines may improve screening. On the other hand, several larger lines in size were recovered in the screening. Because properties of cell ploidy were not largely different between mutants and wild-type, a novel mechanism will improve sizes of these mutants.

### Chapter 6. Differential regulation of root architecture in autotetraploid *Arabidopsis thaliana* under boron deficiency

In the screening performed in chapter 5, three long-root mutants under boron deficiency were isolated. Morphology of one of these mutants, designated as A152B, was similar to tetraploid, such as bigger seeds, bigger flowers, and bigger pollens. To confirm ploidy, cell ploidy was measured in wild-type and A152B. The peak for nuclei containing two sets of chromosomes existent in wild-type was not observed in A152B. Thus A152B was autotetraploid. Root elongation of other autotetraploid lines obtained from stock center was also improved under boron deficiency. Fresh weights of both root and shoot were heavier in tetraploid or triploid under normal condition. Because proportion of fresh weights between diploid and polyploids was not largely altered under boron deficiency, polyploids are not tolerant to boron deficiency but are differentially regulated in their root morphology under boron deficiency. Elongation of root cells is inhibited under boron deficiency. This inhibition was milder in tetraploid, which causes improved root elongation under boron deficiency.

### Discussion

In this thesis, several responses of *Arabidopsis* to nutrient deficiencies and toxicity were revealed. Although the extreme goal of the study was to improve plant tolerance to nutrient

stresses, no novel strategy to attach tolerance to nutrient stresses was identified. Screening of gain-of-function mutants, performed in chapters 5 and 6, was a novel method and I expected to isolate various genes. The failure of the screening indicated incompleteness of the screening and necessity to improve screening systems.

Several nutrients are deficient in some or many crop fields. Fertilizers are added to these fields to support crop production. Nitrogen and phosphorus fertilization can cause enrichment of these elements in environment and each nutrient can threat species richness (Stevens et al. 2004, Wassen et al. 2005). Another point to stress is that some estimations predict depletion of inexpensive phosphorus ores for fertilizer in the world by 2050 (Vance et al. 2003). Improvement of crop uptake and use efficiency of nitrogen and phosphorus is an important approach to settle these severe situations.

The number of endogenous genes which improve plant tolerance to nutrient deficiency is restricted. This may indicate that *Arabidopsis* and other plant species evolved nearly fully to adapt to nutrient deficiencies, and not allowing further tolerance attached by modification of endogenous genes. A recent study in our laboratory, on the other hand, indicates difference between ectopic overexpression under the control of 35S promoter and activation through combination of 35S enhancer and native promoter of an endogenous gene. If this is true to that gene, the same phenomenon could be observed in other genes. My recent trial also supported this phenomenon. Although further confirmations are necessary, these results indicate that tolerance to nutrient deficiency is attached to plants only by activating functionally identified endogenous genes. This may be an important trial for sustainable agriculture.

After identification of genes which improve plant tolerance to nutrient deficiency, can we modify crops without transgenes? We can find an example in which bread wheat was reverse-genetically improved without transgenes by TILLING (Slade et al. 2005). Although mutation is randomly inserted in TILLING and most mutations are restricted to C to T or G to A transitions consistent with guanine alkylation (Colbert et al. 2001, Slade et al. 2005), sequential TILLING and backcrossing may enable modification of promoter sequences or modification of codon usage through accumulation of silent mutations in favor of major codons, for activation of gene expressions. Or alternatively, some transposons enhance expressions of nearby genes or may shut down the spreading of heterochromatins along chromosome from original target sites of transcriptional gene silencing onto the target genes. For application of basic knowledge in plant genetics to improvement of crop tolerance to nutrient deficiency, I find several very challenging but not impossible studies, which should be performed to save our future.

## Publications


Kasajima I, Ide Y, Ohkama-Ohtsu N, Hayashi H, Yoneyama T, Fujiwara T (2004) A protocol for rapid DNA extraction from *Arabidopsis thaliana* for PCR analysis. Plant Mol Biol Rep 22: 49-52

Yoneyama T, Dacanay EV, Castelo O, Kasajima I, Ho PY (2004) Estimation of soil organic carbon turnover using natural C-13 abundance in Asian tropics: A case study in the Philippines. Soil Sci Plant Nutr 50: 599-602

Ohkama-Ohtsu N, Kasajima I, Fujiwara T, Naito S (2004) Isolation and characterization of an Arabidopsis mutant that overaccumulates *O*-acetyl-L-Ser. Plant Physiol 136: 3209-3222

Kasajima I, Ohkama-Ohtsu N, Ide Y, Hayashi H, Yoneyama T, Suzuki Y, Naito S, Fujiwara T (2007) The *BIG* gene is involved in regulation of sulfur deficiency-responsive genes in

*Arabidopsis thaliana*. Physiol Plant 129: 351-363
Kasajima I, Fujiwara T (2007) Identification of novel *Arabidopsis thaliana* genes which are induced by high levels of boron. Plant Biotech 24: 355-360