論文の内容の要旨

応用生命化学　専攻
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論文題目　The differential roles of NLPs in regulating nitrate-dependent growth in rice

（イネの異なる NLP の硝酸依存的な生育における役割）

Introduction

Nitrogen is an essential macronutrient for plants and the limiting factor for growth and development when the supply is not sufficient. Nitrate also works as a signaling molecule which is considered to be at the center of communications between plant intrinsic programs and the environment. Higher plants have evolved several mechanisms to adapt to changes in nitrogen supply and to use a variety of forms of nitrogen. In order to understand the nitrate signaling pathway, the functions of a number of nitrate transporter genes and nitrate assimilation genes have been widely reported. Nitrate transporters can be classified into two main families NRT1 (low-affinity transport system) and NRT2 (high-affinity transport system). Nitrate assimilation are energy-consuming reactions involving the sequential reduction and assimilation of nitrate to nitrite, ammonium, glutamine, and then to glutamate. Nitrate reductase (NR), nitrite reductase (NiR) and glutamine synthetase-glutamate synthase (GS-GOGAT) are responsible for this pathway.

In recent years, NLPs (NIN-like protein) in *Arabidopsis thaliana* are identified as key transcription factors in nitrate signaling, regulating the expressions of a number of nitrate related genes. The RWP-RK domain is most conserved region in NLPs with the function of DNA binding and protein dimerization. AtNLP7 is reported to regulate nitrate responses and works as a regulator of primary nitrate response. AtNLP8 is essential for nitrate-promoted seed germination. More interestingly, the localization of AtNLP7 is regulated by nitrate via a nuclear retention mechanism. The phosphorylation of a conserved Ser205 in AtNLPs by CPKs is necessary to sense nitrate signaling.

Till now, the functions of OsNLP family are not well described. In plants, homologous proteins might have evolved different molecular mechanisms between dicotyledons and monocotyledons. Rice is not only a model plant but also the most important crop in the world. For most crops, N fertilizers is the main effector for annual

yield. Nevertheless, the benefits of N fertilizers added to cropping systems come with noticeable energy and environmental costs. I initiated my Ph.D. study by analyzing *osnlp1*, *osnlp4* and *osnlp1/4* mutants, in order to understand the mechanisms of OsNLP family in rice growth under nitrate condition and provide a new sight on improving rice nitrogen use efficiency in paddy field.

Chapter 1. *OsNLP4* is a key gene regulating growth under nitrate condition in rice

There are six members in rice NLP family. OsNLPs can be classified into three clades (OsNLP1 and OsNLP4, OsNLP2 and OsNLP5, OsNLP3 and OsNLP6). The mRNA expressions of six *OsNLPs* under four different nitrogen conditions (2 mM KNO_3 (nitrate), 2 mM NH_4Cl (ammonium), 1 mM KNO_3 + 1 mM NH_4Cl (normal) and 2 mM KCl (nitrogen free)) were examined and it was found that *OsNLP4* mRNA accumulation was increased significantly under nitrogen free condition, but not under other conditions.

In a Tos-17 insertion line of *OsNLP4* (*osnlp4-1*), *OsNLP4* mRNA accumulation was reduced. The insertion line showed shorter shoot-/root- length and reduced shoot-/root- dry weight compared with wild type (Nipponbare) when nitrate was used as a sole source of nitrogen but not when ammonium was supplied, suggesting that *OsNLP4* plays an important role in nitrate dependent growth in rice. These were reproduced in two CRISPR/Cas9 line of *OsNLP4* (*osnlp4-2* and *osnlp4-3*). In the *osnlp4* lines, mRNA accumulation of the nitrate reductase gene *NIA1* was declined. But mRNA accumulation of nitrite reductase gene *NIR1* was not affected. On the other hand, some genes related to nitrate transport (*NRT2.1* and *NRT1.5a*) had similar mRNA accumulation between wild type and the *osnlp4* mutants. In addition, the total shoot nitrate concentration was reduced to around 20% of WT in *osnlp4* mutants. Furthermore, nitrate uptake rate, total N amount and nitrate reductase activity were also reduced in the *osnlp4* mutants. In rice protoplast transient expression, OsNLP4-GFP signal was detected mainly in the nucleus with minor signals in the cytosol. These patterns of subcellular localization did not seem affected by changing the nitrate condition.

Chapter 2. The differential role of *OsNLP1* in regulating growth under nitrate conditions in rice

Among six members of *OsNLP* family in rice, except *OsNLP4*, the mRNA expression level of *OsNLP1* was also induced significantly only under nitrogen free condition. I examined the growth of a Tos-17 insertion line of *OsNLP1* (*osnlp1*) and a double mutant line (*osnlp1/4*) under different nitrogen conditions (as Chapter 1). The *osnlp1* showed shorter root length and reduced root dry weight as the *osnlp4* when nitrate was used as a sole source of nitrogen. But the shoot part did not show any obvious phenotype compared with WT, unlike the case

of the *osnlp4*. The *osnlp1/4* line showed severer growth defects compared with the *osnlp4* single mutant. Additional low and high nitrate conditions data confirmed that the root part phenotypes of *osnlp1* are induced by nitrate independent of its concentration. Moreover, total nitrate concentration and nitrate reductase activity were decreased in the *osnlp1/4* but not in the *osnlp1*. *In vitro* test, the NiR activity was quit low and there was no significant difference between WT and all mutants. In the *osnlp4*, mRNA accumulation of genes related to nitrate assimilation were reduced, while those of nitrate transporter genes were reduced in the *osnlp1* mutant line. In the way of the double mutant *osnlp1/4*, it was reasonable to see both nitrate assimilation genes and nitrate transporter genes were impacted.

More interestingly, translational fusions with GFP showed that OsNLP1 appeared to be localized to nucleus in a nitrate-dependent manner activated by nitrate-dependent nuclear retention. Under nitrate starvation, signal for OsNLP1 was found in the nucleus with some addition signal outside the nucleus. Under nitrate supply condition, OsNLP1 was located in the nucleus. Additionally, no physical interaction between OsNLP1 and OsNLP4 was observed in my conditions.

On the other side, the phenotypes of *osnlps* mutants were not nitrate-specific. Nitrite and glutamine, the products of nitrate in assimilation pathway, cannot recover the growth defects of mutants totally. All *osnlps* mutants showed similar development defects in agar mediums containing nitrite or glutamine as them in nitrate condition. However, the C/N ratios of the *osnlp4* and *osnlp1/4* were reduced to WT level.

In order to check agronomic traits of our *osnlps* lines, I planted WT, *osnlp1*, *osnlp4* and *osnlp1/4* in fertilization (+N) and non-fertilization area in the paddy field of Tohoku University (Sendai, Japan) in 2018. In fertilization area, there was no significant difference between WT and the *osnlp1* in tiller number, straw weight and panicle weight. Only the *osnlp4* and *osnlp1/4* showed decreased straw weight.

Chapter 3. The phenotypes of overexpression lines of *OsNLP*1, *OsNLP4* and *OsNLP6* in a range of nitrogen conditions

Recently, the homozygous overexpression lines of *OsNLP1*, *OsNLP4* and *OsNLP6* were obtained. The phenotypes of these lines in a range of nitrogen conditions were checked (as Chapter 1). The most interesting thing is that in nitrogen free condition, the growth of all ox-lines was better than WT, which means longer shoots and roots and higher dry weights. The shoot dry weight of the ox-*OsNLP6* line was about 25 mg, almost twice of WT, while the ox-*OsNLP4* line showed the highest root dry weight at around 10 mg. But the difference in C/N ratio among WT and ox-lines was not significant. In nitrogen supply conditions, these ox-lines also had some

different phenotypes with WT. For example, the shorter root of all ox-lines were observed in nitrate condition, though no obvious change was in root dry weight. Absolutely, the phenotypes should be confirmed further using more independent lines or nutrient conditions.

Conclusion

In this research, I have identified the fundamental functions of *OsNLP1* and *OsNLP4* genes and hypothesized the networks of both genes in nitrate signaling pathway. OsNLP1 and OsNLP4 take different roles in regulating rice growth in nitrate condition. The former only functions in root via regulation of some nitrate or nitrite transporter genes and root elongation related genes, while the latter acts mainly in shoot by nitrate assimilation pathway controlling the growth at the whole plant level. In the *osnlp4*, the reduction from nitrate to nitrite is the key inhibited step. The following reactions and GS/GOCAT cycle are also impaired at some extend in both *osnlp1* and *osnlp*4. Through the study on *OsNLP1* and *OsNLP4* genes, all these findings brought together show the importance of NLPs family in nitrate-dependent growth in rice and OsNLPs have separate roles in nitrate signaling or other signaling. In the future, I intend to work on these overexpression lines and construct crispr lines of other *OsNLPs* to extend our understanding of OsNLPs involvement in the nitrate signaling system. The way OsNLPs sensing the nitrate signaling, the binding sites in downstream-regulated genes and the crosstalk with other signaling will also be examined. Our ox-lines indeed showed better growth than wild type in nitrogen free condition. These studies will shed light on improving rice annual yield in field in an environmentally-friendly way.

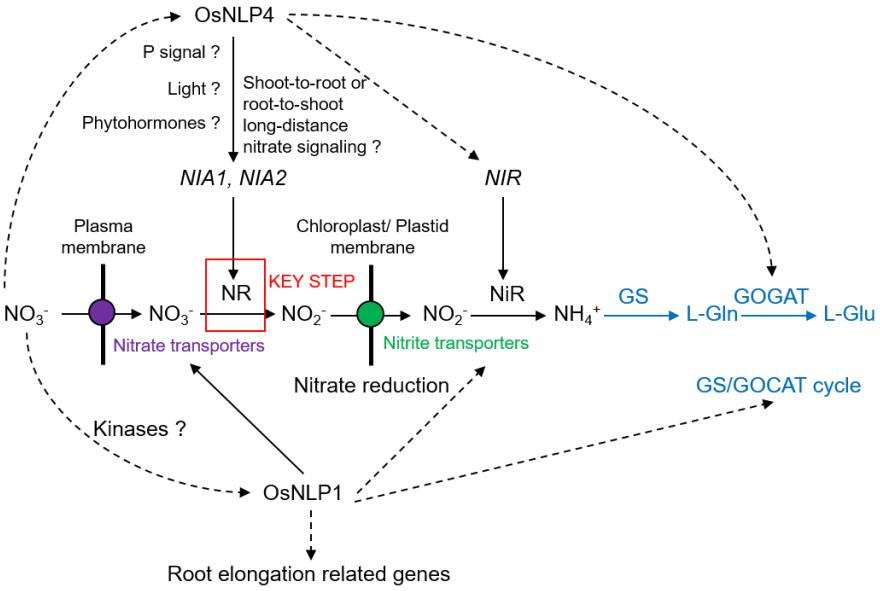

Figure. A schematic model of proposed OsNLP1 and OsNLP4 regulation network in rice.