論文要旨の内容

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論文題目

Roles of NIP genes for Transport and Distribution of Boron in *Arabidopsis thaliana* – Differential Expression and Functions of NIP5;1 and NIP6;1

（シロイヌナズナのホウ素輸送や分配における NIP 遺伝子の役割-NIP5;1 と NIP6;1 の異なる発現制御と機能-）

Boron (B) requirement in plants was first described more than 80 years ago, and B availability in soil is an important determinant of agricultural production. B availability in soil is limited in many regions in the world, including Japan. Understandings of the molecular basis of the requirement and transport are necessary for improvement of agricultural production.

Introduction

B is an essential element for plants. The only function of B in plants proven at the molecular level is borate crosslinking of rhamnogalacturonan-II in cell walls (O'Neill at al., 2001). This crosslinking was shown essential for normal expansion of leaves.

B in neutral solution is mostly present as boric acid (H_3BO_3). Being a noncharged molecule, boric acid has a very high permeability coefficient for lipid bilayer, and it has long been believed that B is transported passively. However, in 2002, BOR1 was identified as a B transporter capable of transporting B against B concentration gradient (Takano et al., 2002). Furthermore, *NIP5;1*, an aquaporin-like gene, which encodes a B channel was found and NIP5;1 facilitated B transport across

the membrane. *NIP5;1* was required for normal plant growth at B deficiency and was upregulated by transcriptional level. *NIP5;1* mRNA accumulation increased by 15-fold in root under the low B condition (Takano et al., 2006).

The primary objective of the present study was to understand physiological function of *NIP6;1*, the most similar gene to *NIP5;1* in *Arabidopsis thaliana*. The regulatory mechanisms of *NIP5;1* in response to B, and differential expression and function of NIP5;1 and NIP6;1 were also studied.

Chapter 1 *Arabidopsis thaliana* NIP6;1 is a boric acid channel that functions in preferential transport of B to young leaves

In addition to being the most similar to NIP5;1, NIP6;1 has identical amino acids in the "selectivity filter". The selectivity filter is amino acid residues shown to be involved in selection of substrates based on the structure and function. To investigate physiological function of NIP6;1 in B transport, *NIP6;1* transcript level, cell and tissue specificity of *NIP6;1*, and plant growth and B concentration of *NIP6;1* T-DNA insertion plants were examined.

NIP6;1 transcript levels in rosette leaves were higher than those in root in contrast to the *NIP5;1* whose transcript accumulates predominantly in roots. *NIP6;1* mRNA accumulation in rosette leaves increased by 1.5-fold under B limitation, while the response was not evident in roots.

To investigate the subcellular localization of NIP6;1, The construct for expression of GFP-NIP6;1 under the control of a cauliflower mosaic virus 35S RNA promoter was introduced into *A. thaliana* plants (*P35S-GFP-NIP6;1*). NIP5;1 is already known to be localized at plasma membrane. *P35S-GFP-NIP5;1* was also introduced into *A. thaliana* plants as a control for a plasma membrane localization. GFP fluorescence in NIP6;1-expressing transgenic plants was localized at the extreme cell periphery with a similar pattern to that in NIP5;1-expressing transgenic plants at the root elongation zone, indicating that NIP6;1 was also localized at the plasma membrane as was the case of NIP5;1.

To verify whether NIP6;1 has a similar B transport property to NIP5;1, NIP6;1 was expressed in *Xenopus oocytes*. These NIP6;1 and NIP5;1-expressing oocytes showed significant swelling upon exposure to boric acid. B concentration determined by using ICP-MS was higher in NIP6;1 and NIP5;1-expressing oocytes than in uninjected oocytes, suggesting that NIP6;1 also facilitates boric acid in *X. oocytes*

To analyze the cell-type specificity of expression of *NIP6;1*, *NIP6;1* promoter fragment containing the 5' UTR fused to the *GUS* reporter gene was introduced into *A. thaliana* plants. Strong

GUS staining was observed in vascular bundles of nodal regions and immature young leaves, but no staining was observed in the mature old leaves. These patterns were identical both under high and low B supply.

To characterize the function of the *NIP6;1* gene in *A. thaliana*, two independent mutant alleles, *nip6:1-1* and *nip6:1-2*, containing T-DNA insertions in the first exon and forth exon of *NIP6;1* coding region, respectively were obtained. At a late vegetative stage, plants supplied with 0.1μM B showed small young leaves in both T-DNA insertion lines compared with that in wild-type (Col-0), whereas the mature leaves of both T-DNA insertion lines were apparently normal at 0.1μM B supply. Both insertion lines grew similar to the corresponding wild-type plants when B was supplied at 100 μM.

B concentrations of young leaves of *nip6;1-1* and *nip6;1-2* plants grown at 0.1 μM B were significantly reduced compared with that of the corresponding leaves in Col-0 plants, whereas concentration in the old leaves were not different between the wild-type and the insertion lines. At 100 μM B supply, there was no significant difference among the lines for B concentrations in both young and old leaves. These data demonstrate that *NIP6;1* is required for expansion of young rosette leaves under low B supply.

Taken together, I demonstrated that NIP6;1 was a functional B channel required for preferential B transport to young leaves under the low B condition

Chapter 2 Promoter analysis of *NIP5;1*, a boron deficiency inducible gene, in *Arabidopsis thaliana*

To clarify *NIP5;1* expression mechanism in response to B, a series of 5' deletions of the upstream promoter region were generated and fused to the *GUS* reporter gene followed by introduction into *A. thaliana* plants.

The GUS expression analysis under normal and B deficiency condition revealed the following

1. Region between-580/-562 is required for expression in root tips under B deficiency.
2. Region between-448/-400 is required for expression in root elongation zone under B deficiency.
3. Region between-400/-300 is required for expression in bulk roots under B deficiency.

In summary, this analysis indicated that B deficiency induction of *NIP5;1* promoter activity is governed at least 3 distinct *cis*-acting elements and each *cis*-acting element is required for upregulation of particular portions of roots. This is the first example of a gene whose nutrient induction is regulated by three distinct *cis*-acting elements in different cell types.

Chapter 3 Isolation of a *NIP5;1*-related boron limitation responseless mutant in *Arabidopsis thaliana*

To identify a key regulatory protein(s) controlling the upstream signaling cascades of *NIP5;1* in response to B, *A. thaliana* mutants were screened.

Seeds of transgenic plants carrying promoter *NIP5;1-GFP* were mutagenized with ethyl methanesulfonate (EMS) and M2 seedlings were used for screening. Thirty thousand M2 seeds were screened for lines with reduced level of GFP fluorescence under the low B condition and eight lines were isolated. Genetic analysis suggests that the phenotype is caused by a single nuclear locus. In the two lines, genetic linkage was found in the downstream of chromosome 5. Fine mapping determined that these mutants were located in 50 kb between the mxk3 marker and mqn23 marker corresponding to the MXK3 and MQN23 clone. To my surprise, in this mutant, *NIP5;1* expression was reduced in root tips but not in the bulk roots. It was concluded that regulation of gene expression in response to B concentration was governed by at least 2 distinct mechanisms, one operating specifically in root tips, the other in the bulk of roots.

Chapter 4 Growth improvement of nip5;1 by disruption of NIP6;1

Both *NIP5;1* and *NIP6;1* encode a boric acid channel, but, as I established above, their roles are different. To verify phenotypes of double T-DNA insertion lines of *NIP5;1* and *NIP6;1*, physiological and molecular analyses were conducted.

Shoot growth in *nip5;1-1* x *nip6;1-1*(referred to as WKO1) and *nip5;1-1* x *nip6;1-2* (referred to as WKO2) mutant plants was higher than that in *nip5;1-1* single mutant plants, but lower than that in *nip6;1-1* and *nip6;1-2* single mutant plants under the low B condition. B concentration in shoots in WKOs mutant plants was higher than that in *nip5;1-1* single mutant plants, but lower than that in *nip6;1-1* and *nip6;1-2* single mutant plants under the low B condition. The mRNA accumulation of *NIP5;1* in *nip6;1-1* and *nip6;1-2* mutant plants was similar to that in wild type, and also the mRNA accumulation of *NIP6;1* in *nip5;1-1* mutant plants was not different from wild type.

Among the phenotypes, it is intriguing to point out that in my system, the WKO plants grow better than the *nip5;1-1* single mutant plants. In other words, mutation in NIP6;1 rescued growth defect of the *nip5;1-1* mutant plants. Considering that both NIP5;1 and NIP6;1 are boric acid channels with different roles in whole-plant B physiology, it is generally assumed that WKO mutants will show severer phenotype than the single mutants. I repeated these experiments 3 times and similar results were obtained in each case. It is not possible to demonstrate mechanisms underlying this phenomenon

at this time, but this is the first example of the rescue of the phenotype by mutation of genes with similar function. The present finding opened a new field of combinatorial genetics and a novel strategy for crop improvement.

Chapter 5 NIP5;1 polar localization and regulatory mechanisms in response to B in *Arabidopsis thaliana*

The construct for expression of GFP-NIP5;1 under the control of the promoter *NIP5;1* was introduced into *A. thaliana* plants (*promoter NIP5;1-GFP-NIP5;1*). GFP fluorescence in the transgenic plants was localized at the distal side of plasma membrane in root epidermis cells.

GFP-NIP5;1 is predominantly accumulate in roots, but not in shoots in *P35S-GFP-NIP5;1* transgenic plants. On the other hand, *GFP-NIP6;1* is accumulated both in roots and shoots in *P35S-GFP-NIP6;1* transgenic plants. Accumulation of *NIP5;1* mRNA was highly increased in roots, but not in shoots in *P35S-GFP-NIP5;1* transgenic plants. In contrast, mRNA accumulation of *NIP6;1* was highly increased both in roots and shoots in *P35S-GFP-NIP6;1* transgenic plants. The promoters used to drive these genes are identical and it is not likely that the difference is due to the promoter activity. These data strongly suggest that accumulation of *NIP5;1* transcript is regulated in among tissues through a post-transcriptional mechanism.

In this chapter I demonstrated two novel regulatory mechanisms for NIP5;1 accumulation. One is distal localization in root epidermis. Considering the physiological role of NIP5;1 as an boron influx channel, it is reasonable that NIP5;1 is distally localized. The second finding is the post-transcriptional regulation of *NIP5;1* transcript accumulation. These finding revealed that NIP5;1 is regulated by not only induction level under B deficiency, but also by mRNA accumulation level and protein level.

Conclusion

Through the analysis of NIP6;1 and NIP5;1, I established novel aspects and mechanisms of B transport in plants. I believe the findings described in the present thesis will be one of the important foundation for future develpoment.

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