博士論文（要約）

New mechanisms of boron utilization during root elongation
-Screening and characterization of boron-sensitive *Arabidopsis thaliana* mutants-

(根の伸長におけるホウ素機能の新しいメカニズムの研究
-ホウ素感受性シロイヌナズナ変異株の単離と解析-)

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論文の内容の要約

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Introduction

Boron (B) has been confirmed as an essential plant mineral nutrition since 1920s. B deficiency and B toxicity in plants are worldwide agricultural problems. To solve these problems, it is important to understand the mechanisms of B absorption/transport and utilization in plants. For the transport, B transport in plants is well-understood through the genetic and physiological analysis of B transporter gene families in *Arabidopsis thaliana*. For the utilization, the only known molecular mechanism is the formation of B-rhamnogalacturonan (RG)-II dimer in plant cell walls. RG-II is a component of pectic polysaccharides. Formation of B-RG-II complex is known to be required for leaf expansion.

A number of evidences point that B regulates the cell division and enlargement through affecting cell membrane, phytohormone, or cell wall polysaccharides besides RG-II. However, the mechanisms for these utilizations are not well described. As a part of efforts to reveal new mechanisms of B utilization, a set of *A. thaliana* mutants that require high concentration of B (high-B), or sensitive to low concentration of B (low-B) in media were isolated in the Laboratory of Plant Nutrition and Fertilizers of the University of Tokyo. I initiated my Ph.D study by analyzing these mutants.

Chapter 1 Boron is required for cellulose biosynthesis and CESA complex movement in *A. thaliana*.

To reveal new mechanisms of B utilization in *A. thaliana*, one of the high-B requiring mutants was molecular genetically characterized. This mutant grows poorly under normal-B supply, and high-B supplement partially recovered the growth. The gene responsible for the phenotype was identified as Chitinase Like 1 (*CTL1)*. B concentration in *ctl1* was identical to that of wild-type, suggesting that *ctl1* was not defective in B transport but was defective in B utilization. The extents of B-RG-II dimerization in *ctl1* were also similar to that of wild-type, suggesting that B-RG-II formation is not the reason for the defects in B utilization. This implies that a new B

function besides B-RG-II formation is present in *A. thaliana* and that CTL1 is required for such function.

For the identification of the new B function in plant growth, it is necessary to identify phenomena that are strongly B-dependent in *ctl1*, but not in wild-type and reproduce such phenomena without using *ctl1* mutation to examine if such phenomena cause B dependency in plant growth. *CTL1* is known to be involved in cellulose biosynthesis. Therefore, I thought that cellulose maybe involved in this new B function. First I examined the B dependency of cellulose contents in *ctl1*. Cellulose in cell wall is present in two forms, crystalline cellulose and amorphous cellulose. The crystalline cellulose contents were less in *ctl1* than that of wild-type under normal-B. High-B treatment significantly increased the crystalline cellulose content of *ctl1* in the hypocotyls, but not in the roots. The amorphous cellulose was highly accumulated in *ctl1* while little accumulated in wild-type irrespective of the B treatment. High-B treatment reduced the accumulation of amorphous cellulose contents in *ctl1*. These analysis revealed that in *ctl1* crystalline cellulose is reduced while amorphous cellulose accumulated to high levels and these trends were less evident under high-B treatment.

CTL1 is also known to affect cellulose synthase (CESA). Cellulose is produced by CESA complexes. The velocity of CESA complexes affects cellulose polymerization. To reveal the effects of B on the polymerization of cellulose in *ctl1*, YFP-labeled CESA6 was used to observe the movements of CESA complexes in live-cells. The velocity of CESA complexes was increased by high-B in *ctl1*, suggesting that high-B accelerates the polymerization of cellulose in *ctl1*.

To examine if B-affected cellulose polymerization is the reason for high-B requirement in *ctl1* growth, the effects of B was examined in mutants with reduced cellulose contents besides *ctl1*. *korrigan* and *cobra* mutants have decreased cellulose content, and the CESA complexes velocity in them are also reduced. These mutants were grown under high-B. High-B partially recovered the root elongation defect of *korrigan* and *cobra*, suggesting that high-B, at least in part, recovers the growth defect of them through enhancing cellulose contents. To further obtain link between B condition and cellulose biosynthesis, a cellulose synthesis inhibitor has been used. I reasoned that if high-B has a positive effect on cellulose synthesis, then effect of cellulose biosynthesis inhibitor maybe weakened by high-B treatment. Isoxaben, a cellulose synthesis inhibitor, reduced cellulose contents and reduce root elongation. The effect of high-B on isoxaben-treated wild-type was examined. High-B partially recovered the root elongation inhibition by isoxaben-treatment. From these results, I concluded that high-B enhances cellulose polymerization which results in recovery of root growth of cellulose synthesis defective mutants and conditions.

Next the effects of B on cellulose biosynthesis was further studied in wild-type. I proposed that B is required for cellulose polymerization also in wild-type. From the analysis above, little amorphous cellulose was in the roots of wild-type. Therefore, only crystalline cellulose was measured. The crystalline cellulose content in the roots in wild-type was significantly decreased under low-B, compared with that of normal-B. To determine the changes on crystalline cellulose contents without chemical treatment, X-ray diffraction was used and it was found that the content of relative crystalline cellulose increased in wild-type with the increase of B concentrations in media. To determine the polymerization of cellulose, the CESA complexes velocity was measured. It was found that the velocity of CESA complexes in wild-type is also increased with the increasing of B content in media.

From all these results, I concluded that B is required for the cellulose biosynthesis through regulating the polymerization of cellulose in the wild type under the normal conditions.

Chapter 2 Boron is also required for structure/formation of non-cellulosic polysaccharides in *ctl1*

In *ctl1*, the contents of non-cellulosic polysaccharides are also affected. Pectic polysaccharide homogalacturonan is increased, and the structure of hemicellulose xyloglucan is changed. I thought that it is also possible that high-B affects the pectic polysaccharides or hemicellulose and that this can also be a part of mechanisms of growth recovery of *ctl1* by high-B treatment. To reveal the roles of B on pectic polysaccharides and hemicellulose, I separated them from the wall by using chemical solvents, respectively. The change of these components was analyzed through determination of relative monosaccharide compositions from pectic polysaccharides and hemicellulose fractions. In the pectic polysaccharide fractions of *ctl1*, compared with that of wild-type, relative content of rhamnose was increased, and those of arabinose and galactose were reduced. High-B treatment partially recovered these changes, revealing that the composition of pectic polysaccharide was affected by *ctl1* mutation and high-B treatment. Arabinose and galactose are the main component of the side chains in pectic polysaccharides RG-I. Therefore, decrease of them suggests that the side chains of RG-I are affected by *ctl1* mutation and high-B treatment. In the hemicellulose fractions of *ctl1*, compared with that of wild-type, relative content of glucose was increased, and that of xylose was decreased. High-B treatment partially recovered these changes, revealing that composition of hemicellulose was affected by *ctl1* mutation and high-B treatment. Xylose is the main component in xyloglucan side chains, and xyloglucan is the dominant hemicellulose in *A. thaliana*. Decreased xylose suggested that side chains of xyloglucan are affected by *ctl1* mutation and high-B treatment. From these results, I found that high-B partially recovered the structure changes of RG-I and xyloglucan in *ctl1*, but whether this is the reason for the high-B requiring root elongation of *ctl1* was still unknown.

Since high-B affected the structures of RG-I and xyloglucan in *ctl1* mutants, I next accessed the effects of B on RG-I and xyloglucan in wild-type under different B conditions. Under both low- and high-B conditions, no significant monosaccharide component change in pectic polysaccharides was found, compared with that of normal-B control. This result suggested that the structure of RG-I is not affected by B in wild-type. For hemicellulose fractions under low-B, but not under high-B, relative content of glucose was increased and that of xylose was decreased, suggesting that the structure of xyloglucan is affected by low-B in wild-type, compared with that of normal-B control. This result also suggests that proper xyloglucan structure is required for low-B tolerance in the wild-type. To investigate the low-B response of mutant with defective xyloglucan structure, the root elongation of *xxt1xxt2* mutant was analyzed. *xxt1xxt2* mutant lacks of xyloglucan side chains. The relative root elongation of *xxt1xxt2* was sensitive to low-B. This is associated with the hypothesis that proper xyloglucan structure was required for low-B tolerance.

Taken all results together, the structures of RG-I and xyloglucan were found to be partially recovered by high-B in *ctl1* mutants. Furthermore, xyloglucan was found to be required for low-B tolerance.

Chapter 3 PIN2, but not PIN1, is critical for root meristem maintenance under low-B conditions in *A. thaliana*

B deficiency causes rapid cessation of root elongation. In addition, B influences auxin transport in plants. To assess the importance of auxin transport in B-dependent root elongation, the B-response of auxin efflux transporter PIN mutants were analyzed.

PIN1-PIN4 are the main PINs that regulate auxin transport in the roots of *A. thaliana*. Therefore, *pin1-pin4* mutants were grown under low-B conditions. Among them, only *pin2* showed a significantly shorter root under low-B than that under normal-B conditions. Moreover, the root meristem size of *pin2* was reduced under low-B conditions. Among the PIN family, both PIN1 and PIN2 are shown to be important for root meristem growth/maintenance under normal conditions. This does not apparently explain why *pin1* and *pin2* mutants exhibited different root meristem phenotype under low-B. To investigate the reason for the differential response of *pin1* and *pin2* mutants under low-B, the effects of low-B on PIN1-GFP and PIN2-GFP accumulation and localization were examined. Low-B did not affect PIN2-GFP, while the accumulation of PIN1-GFP was reduced, suggesting PIN1 specific down-regulation by low-B.

These results indicate that under low-B conditions PIN1, but not PIN2, is downregulated and such regulation creates a situation in which only PIN2 is responsible for root meristem maintenance. This is the most likely reason why *pin2* but not *pin1* is strongly affected by low-B.

Chapter 4 Excess-B affects PIN1/PIN2 subcellular localization and accumulation in *A. thaliana*

In Chapter 3, PIN2 was found to be required for root meristem maintenance under low-B. Excess-B (3 mM B) is reported to inhibit the root elongation through reduction in the meristem size in *A. thaliana*, I thought that it is also possible that PIN2 is involved in meristem size maintenance under excess-B. To access the effect of PIN2 on excess-B tolerance, the root elongation of *pin2* was measured under excess-B. *pin2* exhibited a significantly shorter root under excess-B condition than that under normal-B, indicating that PIN2 is also required for excess-B tolerance.

As shown in Chapter 3, PIN1 and PIN2 are differentially regulated by low-B. To examine if they are also differentially regulated by excess-B, the expression and localization of PIN1-GFP and PIN2-GFP were observed under excess-B. The expression of PIN1-GFP was upregulated, and the endocytic trafficking of PIN1-GFP was disrupted by excess-B. The expression of PIN2-GFP was not changed, but the endocytic trafficking and subcellular localization of PIN2-GFP were disrupted by excess-B. These results suggest that excess-B affects the subcellular localization and accumulation of PIN1 and PIN2.

Conclusion

Prior to my study, B has only been known to be involved in B-RG-II dimerization. In this research, B was firstly demonstrated to be important for the regulation of cellulose biosynthesis, and I also demonstrated that B affects the structures of non-cellulosic polysaccharides. These findings bring us a new understanding of B utilization. My findings may lead to a technology that allows artificial facilitation of cellulose biosynthesis through regulation of B nutrition.

The present thesis also demonstrated that low-B downregulates the accumulation of PIN1, but not PIN2. PIN2 is required for both low- and excess-B tolerance. Excess-B affects the endocytic trafficking and polar localization of PIN1/PIN2 proteins in root meristems. These findings are proposed to explain the reasons of B-affected auxin transport. In the future, a new technology can be created to regulate the root elongation of plants under low-B or excess-B conditions through modulating PIN1/PIN2.