論文の内容の要旨

応用生命工学専攻
平成 20 年度博士課程　進学
氏名　坂本卓也
指導教員名　藤原徹

論文題目

**Study of the molecular mechanisms of boron toxicity in plants**
**-Characterization of *Arabidopsis thaliana* mutants hypersensitive to excess boron-**
(植物におけるホウ素毒性の分子機構の研究
-ホウ素過剰超感受性シロイヌナズナ変異株の解析-)

The molecular mechanisms of boron (B) toxicity are not well-understood. In order to obtain insights into the molecular mechanisms of B toxicity, seven *Arabidopsis thaliana* mutants hypersensitive to excess B (*heb*) were studied. Through the analysis of the mutants, I identified six genes involved in B toxicity tolerance. These genes represent the first identification of genes essential for B toxicity-tolerance in plants.

**Introduction**

Boron (B) is an essential element for plants. It can also become toxic when it exists in soils at excessive levels. Limitation of crop yield and quality caused by B toxicity is an agricultural problem in the world especially in semi arid areas.

To understand B toxicity mechanisms and breed excess-B tolerant crops, isolation of genes involved in B toxicity and/or tolerance has been attempted. Recently, overexpression of B transport molecules BOR4 and TIP5;1 were revealed to improve excess-B tolerance in plants, although the biological functions of these molecules in B toxicity have remained unclear. These studies established that regulations of molecules function in efflux and uptake of B are major mechanisms for excess-B tolerances in plants. On the other hand, at the molecular level, mechanisms of excess-B toxicity are still unknown. Other than B transport molecules, several *A. thaliana* proteins involved in transcription, RNA process and anti-oxidative system are shown to provide B tolerance to yeast. However, their functions in B toxicity-tolerance are not revealed yet. Moreover, it has not been elucidated whether these genes are essential for B toxicity-tolerance in plants. Isolation and identification of novel plant genes involved in B toxicity and/or tolerance is expected to provide us new insights into molecular

mechanisms of B toxicity in plants.

For this purpose, I focused on genetic approach using EMS mutagenized *Arabidopsis thaliana* (ectype Col-0). I studied seven recessive mutants, *hypersensitive to excess B* (*heb*). The *heb* mutants showed extremely shorter relative root length than the wild type under the toxic B condition (3 mM boric acid), although they showed slightly reduced root elongation under the control condition (0.03 mM boric acid), indicating their hypersensitivity to excess B. In the present thesis, I first identified the genes that are essential for B toxicity-tolerance in plants using the *heb* mutants and characterized their functions in B toxicity-tolerance. Through the analyses, I established new aspects of two protein complexes, condensin II and 26S proteasome.

## Chapter 1 Condensin II alleviates DNA damage and is essential for excess boron tolerance in *Arabidopsis thaliana*

First I investigated the mineral specificity of the short-root phenotype of *heb1-1* and *heb2-1*. The root growth of *heb1-1* and *heb2-1* were not sensitive to B deficiency, arsenite toxicity and salinity stress, indicating that short-root phenotype of *heb1-1* and *heb2-1* are specific to excess B among mineral stresses tested.

Genetic mapping and sequence analysis revealed that *heb1-1* carried two mutations in At1g64960 which encodes chromosomal associated protein-G2 (CAP-G2) and that *heb2-1* carried a mutation in At3g16730 encoding CAP-H2. Introduction of GFP-fused CAP-G2 and CAP-H2 into the respective *heb* mutants complemented their excess-B dependent phenotype, confirming that these are responsible genes of the *heb* mutants. Both proteins are subunits of chromosomal protein complex condensin II, suggesting that the function of condensin II complex is crucial for excess B tolerance in *A. thaliana*.

Condensin II is composed of two core subunits CAP-C and CAP-E and three regulatory subunits HEB1/CAP-G2, HEB2/CAP-H2 and CAP-D3. In human cells, condensin II is well known to have a role in mitotic chromosomal condensation in concert with another type of condensin, condensin I. In human cells, in addition to the mitotic function, condensins are known to be involved in DNA damage repair during interphase. To investigate whether *A. thaliana* condensin II is involved in DNA damage response as is the case in animal cells, I examined the sensitivity of *HEB1/CAP-G2* and *HEB2/CAP-H2* mutants to reagents/conditions that induce DNA double strand breaks (DSBs). The root growth of both *heb1-1* and *heb2-1* were sensitive to DSBs-inducible reagents compared to the wild type, suggesting the involvement of condensin II in DNA damage repair and/or in resistance to genotoxicity.

To examine whether excess B causes DNA damage, I investigated the expression of DSBs-inducible genes and the levels of DSBs in the root tip cells treated with excess B. RT-PCR revealed that expressions of DSBs-inducible genes such as *BRCA1* and *PARP1* were up-regulated by excess B treatment in both the wild type and the mutants. Transcripts of these genes were higher in the *heb* mutants than in the wild type both under the control and the excess B conditions. Comet assay revealed that the *heb* mutants highly accumulated DSBs compared to the wild type under the control and the excess B conditions. The levels of DSBs in both the wild type and the *heb* mutants were elevated by the excess B treatment. Taken together, these results demonstrate that excess B

causes DSBs in root tip cells and *A. thaliana* condensin II has a role in the alleviation of DNA damage.

In conclusion, I demonstrated that involvement of DSBs in B toxicity and a novel function of plant condensin II in repairing damaged DNA and/or protecting genome from genotoxic stresses especially under the excess B condition.

**Chapter 2 Involvement of 26S proteasome in excess B tolerance in *Arabidopsis thaliana*-Identification of possible targets involved in excess B tolerance-**

Genetic mapping and sequence analysis revealed that *heb3* carries a mutation in At5g05780 which encodes regulatory particle non-ATPase 8a (RPN8a) and *heb6* in At3g05530 which encodes regulatory particle triple-A-ATPase 5a (RPT5a). *heb7* had a mutation in RPT5a at a different site from *heb6*. These mutants were not much sensitive to B deficiency, cadmium, arsenite and sodium chloride toxicity as compared to excess B, indicating the specificity of the mutants to excess B tolerance. To avoid confusion, I renamed *heb3*, *heb6* and *heb7* as *rpn8a-2*, r*pt5a-5* and *rpt5a-6*, respectively.

RPN8a and RPT5a are subunits of 19S regulatory particle (RP) of 26S proteasome (26SP), a large proteolytic device. RP functions in recognition and unfolding of target proteins which are mostly modified by ubiquitin (Ub). In *A. thaliana*, most of the RP subunits were encoded by two genes, suggesting a diverse subunit combination of 26SP is present, which may expand the target specificity and functions. Among T-DNA inserted RP mutants I examined, *rpn2a* and *rpt2a* mutants were also hypersensitive to excess B, but *rpn2b*, *rpn8b*, *rpt2b* and *rpt5b* mutants were not. This suggests the existence of specific combination of RP subunits and specific targets in response to B toxicity.

I elucidated whether the total Ub-dependent proteolysis activity is reduced in the *rpt5a* mutants. The *rpt5a* mutants were sensitive to treatment with amino acid analogue which induces accumulation of misfolded proteins. Misfolded proteins are known to be degraded through the Ub-26SP pathway. This suggests the reduced total Ub-dependent activity in the *rpt5a* mutants and that excess B may cause protein misfolding. On the other hand, the levels of accumulated poly-ubiquitinated proteins in the *rpt5a* mutants were not higher than those in the wild type under the normal B condition. This indicates that the accumulation of poly-ubiquitinated proteins does not reflect the reduced total Ub-dependent activity in the *rpt5a* mutants. Interestingly, the accumulations of poly-ubiquitinated proteins were increased by excess B in the *rpt5a* mutants, but not in the wild type. Taken together, these data suggest that RPT5a contained 26SP is involved in the degradation of those proteins induced by excess B.

To investigate whether the subunit specific poly-ubiquitinated proteins in response to excess B are present, I conducted proteome analysis of poly-ubiquitinated proteins. Poly-ubiquitinated proteins were purified from the root extracts of the wild type and *rpt5a-6* and were analyzed by isobaric tag for relative and absolute quantification (iTRAQ) LC–MS/MS. As a result, 30 of 57 identified proteins were relatively quantified. Accumulations of 21 of 30 proteins were higher in *rpt5a-6* than in the wild type irrespective of B condition and were elevated by excess B treatment, suggesting that those proteins are degraded through a pathway that requires RPT5a. Some of the identified proteins were known to associate to stress response and cell morphogenesis. One possibility is that these proteins undegraded are cause of excess B sensitivity.

As another approach to elucidate molecular mechanisms of RPT5a involvement in excess B

tolerance, several revertants carries *rpt5a-6* mutation and can elongate roots under the excess B condition were isolated. The revertants are expected to provide molecular information on the function of RPT5a in tolerance to excess B.

In conclusion, in this chapter, I demonstrated the requirements of RPN2a, RPN8a, RPT2a and RPT5a for B toxicity-tolerance. I propose that among a variety of compositions of 26SP, those containing RPN2a, RPN8a, RPT2a and/or RPT5a are crucial for excess B tolerance. These sets of 26SP may have essential function in B toxicity-tolerance through Ub-dependent proteolysis of certain proteins with negative effects on root growth.

## Chapter 3 *Arabidopsis thaliana* 26S proteasome subunits RPT2a and RPT5a are crucial for Zinc deficiency-tolerance

Through the analysis of nutritional response of RP mutants, I found that the shoot growth of *rpt2a* and *rpt5a* mutants were sensitive to zinc (Zn) deficiency.

I first speculated that the *rpt* mutants are defective in the regulation of Zn uptake. However, in the *rpt* mutants, shoot Zn contents were similar to that of the wild type. On the other hand, transcripts of Zn deficiency-inducible genes, *ZIP4* and *ZIP9* were highly accumulated in the *rpt* mutants, suggesting the possibility that the *rpt* mutants are suffering from various Zn deficiency symptoms although Zn levels are not reduced.

Indeed, lipid peroxidation levels, known to be increased under Zn deficiency, were higher in the *rpt* mutants than in the wild type, suggesting that ROS accumulation in the *rpt* mutants are higher than in the wild type.

It has been known that up-regulation of 26SP subunit genes reflects the decrease in Ub-dependent 26SP activity in plants. Zn deficiency induced expression of both *RPT2a* and *RPT5a* genes, and the extents of induction of these genes were much higher in the *rpt* mutants, suggesting the reduced activities of Ub-dependent proteolysis under Zn deficiency, especially in the *rpt* mutants. Indeed, poly-ubiquitinated proteins were accumulated upon exposure to Zn deficiency, especially in the *rpt* mutants.

Overall, my analysis established that RPT2a and RPT5a are involved in Zn deficiency response, possibly through alleviation of oxidative stresses and/or processing of poly-ubiquitinated proteins.

## Conclusion

Through the characterization of *heb* mutants, I identified six genes required for B toxicity-tolerance in plants and established novel aspects and mechanisms of B toxicity at the molecular level. In addition, the present thesis also provides novel aspects of condensin II and 26S proteasome function in nutritional responses.

## References

Sakamoto T, Kamiya T, Sako K, Yamaguchi J, Yamagami M and Fujiwara T. *Arabidopsis thaliana* 26S proteasome subunits RPT2a and RPT5a are crucial for Zinc deficiency-tolerance. *Bioscience, Biotechnology, and Biochemistry* ***accepted***.