論文の内容の要旨

応用生命工学専攻
平成16年度博士課程　進学
氏　名　　三輪　京子
指導教員名　小柳津　広志

論文題目

Generation of Boron Stress-Tolerant Arabidopsis -Characterization and Modulation of BOR transporters-

(ホウ素ストレスに耐性なシロイヌナズナの作出 -BOR 輸送体の解析と応用-)

Boron (B) was established more than eighty years ago as an essential trace element for higher plants. Both B deficiency and toxicity have negative effects on plant growth and development. As results, the quality and quantity of agricultural production are hampered in soils with low or excess B, which are widely distributed over the world. Understanding the molecular mechanisms of B transport and B utilization is expected to assist in the alleviation of these problems. The present thesis describes successful generation of the transgenic *Arabidopsis thaliana* plants tolerant to B deficiency and toxicity by overexpessing BOR transporters.

Introduction

A number of physiological studies have suggested that B deficiency affects the growing portions of plants. The symptoms of B deficiency include the cessation of root elongation, reduced leaf expansion, and the loss of fertility. At present, there is compelling evidence that the crosslinking of rhamnogalacturonan-II (RG-II) in cell walls is a primary physiological role of B in plants. BOR1 was identified as the first B transporter in biological systems through the analysis of the *A. thaliana bor1-1* mutant (Takano et al., 2002). BOR1 encodes an efflux-type B transporter required for xylem loading of B under limited supply of B. It was demonstrated that BOR1 was regulated at posttranscriptional level in response to B status. In the transgenic plants expressing BOR1-GFP under the control of the cauliflower mosaic virus (CaMV) 35S RNA promoter, BOR1-GFP accumulated at plasma membrane under low B supply and was degraded via endocytosis upon high B supply. These results indicate that *A. thaliana* plants have mechanisms to sense B conditions in environment and regulate BOR1 accumulation (Takano et al., 2005). Excess B is also known to damage plant growth, causing necrosis in marginal part of leaves and reduced root elongation. Physiological studies have suggested that B exclusion from roots is one of B-tolerant mechanisms, however, no genes responsible for high B tolerance in plants were identified.

The objectives of the present study were to understand molecular mechanisms on B transport and develop the transgenic plants tolerant to B nutrient disorder.

Chapter 1 Expression and function of *BOR1* paralogs in B transport in *Arabidopsis thaliana*

To describe whole molecular mechanism of B transport in plants, expression and function of six *BOR1* paralogs present in the genome of *A. thaliana* were investigated.

First, full length cDNAs corresponding to all the six genes (*BOR2-7*) were obtained. To examine B transport activity, BOR2, BOR3, BOR4, BOR5, and BOR6 were expressed in yeast lacking ScBOR1, the yeast B transporter. The B concentration of yeast cells were decreased in cells expressing any of the BOR genes, compared to the yeast carrying an empty vector. It was established that these paralogs encode functional efflux type B transporters, as is the case of BOR1.

Accumulation of mRNAs of *BOR2*, *BOR3*, *BOR4* and *BOR5* were detected by RT-PCR both in shoots and roots of plants at vegetative stages, however, *BOR6* and *BOR7* transcripts were detected only in flowers. For analysis of cell type specificity of expression, transgenic *A. thaliana* lines expressing GUS or GFP under the control of promoter of each gene were generated. It was found that *BOR2* was predominately expressed in root elongation zone and cortex cells of mature portion of the roots, *BOR3* in cortex cells in roots, guard cells in shoots, *BOR4* in endodermis in roots, *BOR5* in epidermis at root tips, stele in roots, and trichomes in leaves, *BOR6* and *BOR7* in mature pollens and pollen tubes. It was established that each *BOR1* paralog has a distinct pattern of cell-type specific expression.

To examine subcellular localization, BOR2-GFP fusion protein was transiently expressed in onion epidermal cells. GFP fluorescence was observed in periphery of the cells, while GFP fluorescence from free GFP was observed in cytoplasmic strands and nucleus. This result suggests that BOR2-GFP is a plasma membrane protein, as is the case of BOR1.

To investigate effects of the loss of function of these genes, physiological properties of T-DNA insertion mutants was examined. *bor2-1* and *bor2-2*, two independent *A.thaliana* lines carrying T-DNA in the 5th and 10th exons of *BOR2*, showed inhibition of root cell elongation under the limited supply of B. Reduction in shoot growth was also observed in *bor2-1* and *bor2-2*, however, the extent of growth reduction was not as severe as *bor1-3*, a T-DNA insertion line of *BOR1*. Severer growth retardation both in roots and shoots was observed in *bor1-3/bor2-1*, the double T-DNA mutant of *BOR1* and *BOR2*, than the single mutants under B limitation. All of these lines grew normally when they were supplemented with the normal level of B. These results suggest that BOR2 is required for normal growth under B deficiency and has at least in part distinct function from BOR1. B concentrations in roots in *bor2-1* and *bor2-2* were not different from that of the wild type plants, however, formation of RG-II-B dimer were decreased in these mutant plants under low B supply. These observations suggest that borate transport by BOR2 from symplasts to apoplasts is required for effective formation of RG-II-B dimer and promotes root cell elongation under B limitation.

T-DNA insertion mutant for *BOR3* did not show an apparent phenotype, but *bor1-3/bor2-1/bor3-1*, a triple T-DNA insertion mutant line, showed more severe root growth retardation under B deficiency compared to the double mutant *bor1-3/bor2-1*, suggesting that BOR3 has a supportive role to BOR1 and BOR2 for delivering B to the site of requirement in roots.

Taken together, it was demonstrated in this chapter that 1) *BOR1* paralogs encode functional efflux-type B transporters, 2) they have different patterns of cell-specific expression, 3) BOR2 functions for root cell elongation under low B supply. The study of BOR2 revealed a new B transport process through which borate transporter locally transports B to cell walls for efficient use of B.

Capter 2 Isolation and characterization of a novel *Arabidopsis thaliana* mutant with altered B nutrition response

To identify new genes responsible for B transport and responses, *A. thaliana* mutants were screened. Two strategies for mutant isolation were undertaken: (1) Isolation of new mutant plants sensitive to B deficiency, which possibly includes mutant not accumulating BOR1 under low B condition, (2) Isolation of mutant plants which accumulates BOR1 under high B condition.

With the first strategy, mutant plants showing inhibition in expansion of upper leaves only under the condition of limited B supply (0.03 μM boric acid) were established. Since the phenotypes searched were similar to that of *bor1-1*, transgenic plants carrying another copy of the *BOR1* gene was used as a parental line to avoid selection of *bor1* allele and to facilitate isolation of new loci. From 10,000 EMS-treated M2 seeds, nine plant lines were isolated. In one mutant line, in which genetic linkage was found in the upstream of chromosome 4, a nucleotide substitution (C245T) leading to amino acid substitution (A82V) in NIP5;1 (At4g10380) ORF was found. During the course of my study, it was demonstrated that NIP5;1 is a boric acid channel required for normal Arabidopsis growth under B limitation (Takano and Wada et al., 2006). It is likely that the mutation in NIP5;1 in this mutant is the cause of the phenotype. The Ala^{82} residue is predicted to be located in the first transmembrane domain and is conserved among NIP family present in the *A. thaliana* genome. This mutant represents the first experimental evidence for the importance of the Ala residue for the function of NIP5;1. Identification of mutation in the *NIP5;1* in the isolated mutant also established feasibility of the screening method.

In the second strategy, promoterCaMV35S-BOR1-GFP transgenic plants were used as a parental line to detect BOR1 accumulation by GFP fluorescence. In the parental line, GFP fluorescence was decreased under high B condition. Twenty thousand EMS-treated M2 seedlings were screeded for plants showing GFP fluorescence under 30 or 100μM boric acid. After the second round of screening, GFP-accumulating phenotype and 3:1 segregation was confirmed in 21 lines. Identification of genes required for B-dependent BOR1 accumulation may lead to identification of sensor molecules for plant nutrient if any, which perceive B nutrient status. It may also uncover molecular mechanism of B-dependent intracellular trafficking of transporters.

Chapter 3 Improvement of seed yields under boron–limiting conditions through overexpression of BOR1, a boron transporter for xylem loading, in *Arabidopsis thaliana*

To improve plant growth under B deficiency through modulation of BOR1 expression, the transgenic *A.thaliana* lines expressing BOR1 under the control of CaMV 35S RNA promoter were generated. In four independent transgenic plants overexpressing BOR1 or BOR1-GFP, root-to-shoot translocation of B was enhanced and shoot growth was greater under B-limiting conditions, as compared to wild-type plants. The transgenic BOR1 overexpressors showed increased translocation of B, especially to the shoot apex, and set seeds normally supplemented with 0.5 μM boric acid, B-limiting conditions, under which wild-type plants failed to set seeds (Figure 1). It is likely that overexpression of BOR1 enhanced endogenous BOR1 function, and increased B translocation results in improvement of growth. In addition, detrimental effects on plant growth were not found under B excess condition in the BOR1-overexpressed lines, assumingly due to degradation of BOR1 under high B supply. **This is the first report of plants that show improved seed**

Figure 1 Fertility of wild type plants and the transgenic plants overepxressing BOR1 (BOR1 OX) grown supplied with 0.5 μM boric acid.

yields under nutrient-deficient conditions as a result of increased production of an essential mineral nutrient transporter.

Chapter 4 Overexpression of BOR4 improved high-B tolerance in *Arabidopsis thaliana*

To examine involvement of BOR against B toxicity, the transgenic plants expressing BOR4 under the control of CaMV 35S RNA promoter was generated. Seven independent transgenic lines overexpressing BOR4-GFP, homozygous for a T-DNA insertion were established. Immunoblot analysis with anti-GFP antibody revealed that BOR4-GFP accumulation was not greatly changed irrespective of B nutrient conditions in the CaMV 35S-BOR4-GFP transgenic lines. This was clearly different from the case of BOR1, which is degraded upon high B supply. In the transgenic lines, GFP fluorescence was predominately observed at periphery of the cells under any of B conditions, indicating that localization of BOR4 at plasma membrane did not respond to B conditions.

All of the seven independent transgenic CaMV 35S-BOR4-GFP lines tested showed improvement of growth compared to the wild type plants when they were grown in the solid media supplemented with toxic level of boric acid (more than 3 mM). More than 6 mM boric acid supply was lethal to the wild type plants, however the transgenic lines (line 4, 12) showing highest BOR4-GFP expression expanded normal green rosette leaves and elongated roots under 10 mM boric acid supply (Figure 2). The transgenic lines did not show any significant difference in growth in the medium containing 30 μM boric acid, normal B conditions.

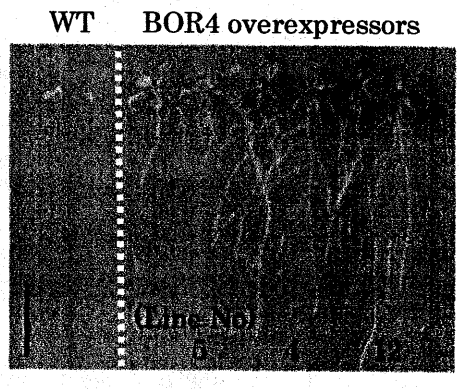

Figure 2 Wild type plants and the transgenic plants overexpressing BOR4 grown under 10 mM boric acid supply (Bar shows 10mm).

These results indicate that BOR4 has a distinct character from BOR1 in terms of protein accumulation and is capable of conferring extremely high B tolerance to *A. thaliana* possibly by B exclusion from roots. **This is the first identification of a gene functioning for high B tolerance in plants, and the first success in generation of the transgenic plants tolerant to B toxicity.**

Conclusion

The present thesis identified efflux-type B tranpsporters and proposed previously unknown processes of B transport in plants. It was demonstrated that BOR2 contributes root cell elongation under B limitation, and BOR4 functions against B toxicity. The finding that BOR4 has a distinct character from BOR1 gives us useful information to investigate molecular mechanism of B-dependent BOR1 endocytosis. The transgenic plants tolerant to B deficiency and toxicity were generated by overexpression of BOR1 and BOR4, respectively. These achievements show significance of BOR transporters to regulate B nutrient condition for plants and provide an effective way to develop crops tolerant to nutrient disorder.

Takano, J., Noguchi, K., Yasumori, M., Kobayashi, M., Gajdos, Z., Miwa, K., et al. (2002) *Nature* 420: 337–340

Takano, J., Miwa, K., Yuan, L., von Wire´n , N., Fujiwara, T. (2005) *Proc. Natl. Acad. Sci. USA* 102:12276–12281

Miwa, K., Takano, J., Fujiwara,T. (2005) *Plant nutrition for food security, human health and environmental protection.*124–125

Miwa, K., Takano, J., Fujiwara, T. (2006) *The Plant Journal*, 46: 1084–1091