Optimisation of nutrient transport processes by plants -boron transport as an example

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Abstract
Boron is an essential micronutrient for plants and is also toxic in high concentrations. In a number of countries, boron deficiency and toxicity hamper agricultural production. One of the strategies to overcome the problem is to improve boron transport/utilization properties of crop plants. For this it is important to understand boron transport mechanisms at the molecular level. Boron transporters were not known until several years ago. We identified BOR1 as the first boron transporter required for efficient xylem loading of boron (B). Arabidopsis and rice have seven and four BOR1 or BOR1-like genes, all likely to encode efflux transporters, with different physiological functions and location within the cell. We also identified NIP5;1, a protein similar to aquaporin, as a transporter required for efficient B uptake. Expression of BOR1 and NIP5;1 is both upregulated under B deficient conditions but with different mechanisms. BOR1 accumulates in plasma membrane under low B conditions, and degraded through endocytosis under sufficient B supply. NIP5;1 is transcriptionally upregulated under low B conditions. Recently we have been successful in generating transgenic plants that are tolerant to low or high boron. In rice, OsBOR1 is required for efficient boron uptake and growth under B-limitation.

Key Words
Nutrient transporters, boron, growth improvement, NIP, BOR, transgenic plants.

Introduction

The essentiality of boron in plants was first described in 1923 (Takano *et al.* 2008, Miwa *et al.* 2009 for review). One of the roles of boron in plants was identified through biochemical and molecular biological analysis to be the crosslinking of pectic polysaccharides, rhamnogalacturonan II (RG-II) molecules. Boron is likely to have other roles as it has been demonstrated that animals and bacteria, that do not have RG-II also require boron. In agriculture, boron deficiency is a major problem that delays crop growth. Typical symptoms of boron deficiency include rapid cessation of root elongation, reduced leaf expansion and reduced fertility. These are mainly due to reduced cell expansion and this corresponds to the roles of boron in cell wall. Boron toxicity is also an important issue. Soils with toxic levels of boron are distributed in arid or semi-arid area and it also affects food production. In terms of crop production, boron toxicity is more difficult to manage than boron deficiency. Boron deficiency can be managed by appropriate boron fertilization. Searches for wheat and barley cultivars that tolerate high levels of boron have long been going on with moderate success. It may require a long term effort, but understanding at the molecular level of the boron transport/utilization mechanisms in plants may provide us with a novel strategy to improve crop plant for better nutrient transport properties that allows plants to withstand low and/or high boron stresses.

Methods

Identification of boron transporters
Arabidopsis thaliana was used for the identification of boron transporters. Two strategies are employed. One is so-called forward genetics. Mutants of *A. thaliana* with altered properties of boron transport/utilization have been screened. Identified mutants were subjected to genetic analysis and with the use of molecular markers, region of the chromosomes where the responsible genes are located are identified. Usually we analysed about 2,000 F2 plants and the regions of chromosomes are narrowed to less than 50 kB before determination of nucleotide sequences. Once the mutation was identified, alleles are obtained in many cases from the ABRC stock centre and used for confirmation of the mutation. Another approach is microarray analysis of the transcripts. RNAs were isolated from boron deficiency treated roots of *A. thaliana* and subjected to Affimetrix microarray analysis. Genes that are strongly induced by low boron treatment were identified and subjected to further molecular genetic and physiological analysis.

Physiological and molecular biological characterisations
Detailed methods are described in our publications. In brief, growth of the mutant plants were analysed in many case on media with altered boron concentration (please see Noguchi *et al.* 1997 for details). Boron concentrations in plant tissues are determined with inductively coupled plasma-mass spectrometry. We have used reporter genes such as β-glucuronidase (GUS) and green fluorescent protein (GFP) for monitoring expression of genes in transgenic plants. Transformation of *A. thaliana* was done with the floral dip method and several independently transformed plants were analysed to confirm functions of introduced transgenes.

Results
BOR1, the first boron transporter identified in the living systems, for xylem loading of boron
A. thaliana bor1-1 mutant was identified as a low boron-sensitive mutant. In *bor1-1*, leaf expansion of upper leaves was inhibited when plants were grown with 3 μM boric acid, while the wild type plants grew normally at this B concentration. Growth of the *bor1-1* plants was similar to the wild type when grown with 30 μM boric acid (Noguchi *et al.* 1997). In *bor1-1* reduction of B concentration was evident in shoots compared to the roots, indicating that the *bor1-1* mutant is defective in B translocation from roots to shoots. Further physiological analysis revealed that the mutant is incapable of concentrating boric acid at the xylem-loading step (Takano *et al.* 2002). In addition to xylem loading, Takano (2001) demonstrated that *bor1-1* is defective in preferential B translocation into young leaves, this may be related to the function of NIP6;1 as described below (Tanaka *et al.* 2008).

Map-based cloning identified *BOR1* as At2g47160. BOR1 has a high similarity to anion exchanger proteins, including the well-characterized Band3 protein in erythrocyte in animals. BOR1 has putative ten transmembrane regions and GFP fusion protein was localized to the plasma membrane. BOR1 has an efflux transport activity of boron in yeast cells (Takano *et al.* 2002). Taken together, BOR1 is an efflux transporter of boron and required for xylem loading of boron from symplasts against concentration gradient. *BOR1*-like genes are also found in yeast and human and some of them are shown to be B transporters (Takano *et al.* 2007). In rice, it is demonstrated that BOR1 is also important for boron transport. Interestingly rice BOR1 not only functions in xylem loading of B, but also boron uptake into roots (Nakagawa *et al.* 2007). This may be a reflection of different root structures and different cell-specificity of expression.

It is known that expression of nutrient transporters are regulated in response to the availability of substrate. In the case of BOR1, mRNA was not significantly changed under various B conditions, however, accumulation of BOR1 protein decreased under high B supply both in roots and shoots (Takano *et al.* 2005). Cell biological analysis including inhibitors of membrane trafficking revealed that BOR1 degradation in response to high boron supply is via endocytosis (Takano *et al.* 2005). This was the first example of an endocytosis-mediated degradation of a plasma membrane protein in plants. This degradation is probably important to avoid high accumulation of boron in shoots under conditions of high boron supply to avoid toxicity symptoms in leaves.

BOR4 for boron exclusion from roots
Six BOR1 paralogs (BOR2-BOR7) are present in the *A. thaliana* genome. We examined transport activities of these paralogs and all were capable of reducing boron concentration in yeast cells. Accumulation of mRNAs corresponding to BOR2, BOR3, BOR4 and BOR5 was detected by RT-PCR both in shoots and roots of plants at vegetative stages. BOR6 and BOR7 transcripts were detected only in flowers. Genetic analysis revealed that BOR2 and BOR3 are important for normal growth under low B condition. BOR4 was found to be involved in high boron tolerance by excluding boric acid out of root cells. BOR4 is an active efflux transporter of boron and is localized in the outer side of the epidermal plasma membrane (Miwa *et al.* 2007).

NIP5;1 for boron uptake into roots
A gene was identified as a low-B inducible gene through the microarray analysis. RNAs were isolated from wild type *A. thaliana* roots treated with normal or low B for 3 days and microarray analysis identified *NIP5;1* as a low-B inducible gene. GFP-NIP5;1 fusion protein was localized to the plasma membrane in *A. thaliana* protoplasts. NIP5;1 allowed high uptake of B into Xenopus oocytes, suggesting that NIP5;1 is a channel for boric acid. The two independent T-DNA insertion lines *nip5;1-1* and *nip5;1-2* showed severe growth reduction both in shoots and in root cell elongation only under limited supply of B, and both grew normally under normal B conditions. Amounts of B uptake into roots were increased in wild type plants

under low B supply compared to those under high B supply, whereas this increase of B uptake was not observed in the *nip5;1-1* mutant. These observations demonstrated that NIP5;1 is essential for B uptake into root cells to support normal plant growth under B limitation. Given the similarity to aquaporins, NIP5;1 is likely to transports boric acid according to concentration gradient, and to contribute to satisfy B requirement in shoot and root growth.

NIP6;1 for preferential distribution of boron to young portions of shoot
We investigated the function of *NIP6;1*, the most similar gene to *NIP5;1*, through reverse genetic approach. NIP6;1 facilitates permeation of boric acid across the membrane when expressed in Xenopus oocytes, but is completely impermeable to water. NIP6;1 transcript accumulation is elevated in response to B deprivation in shoots, but not in roots. *NIP6;1* promoter-GUS (Tanaka *et al.* 2008) is predominantly expressed in nodal regions of shoots, especially the phloem region of vascular tissues. Three independently identified T-DNA insertion lines for the *NIP6;1* gene exhibited reduced expansion of young rosette leaves only under low B conditions. B concentrations decreased in young rosette leaves but not in the old leaves of these mutants. These results strongly suggest that NIP6;1 is a boric acid channel required for proper distribution of boric acid particularly to young developing shoot tissues. NIP6;1 is likely to be involved in xylem-phloem transfer of boric acid at the nodal regions. The water-tight property of NIP6;1 may be important for the boric acid transfer without disturbing transport processes in phloem.

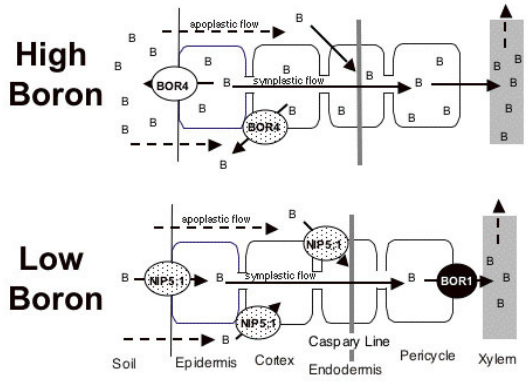

Figure 1. A model of boron transport across root cells in *Arabidopsis thaliana*. Under low boron conditions, NIP5;1 and BOR1 coordinately drives efficient boron transport across roots into xylem. Under high boron conditions, NIP5;1 is not strongly expressed and BOR1 degraded. Instead, BOR4 is accumulated to drive efflux of boron from symplasm to soil solution to reduce concentration of toxic boron in roots. Role of NIP6;1 is mostly in shoots.

Generation of plants tolerant to boron stresses through enhancement of boron transporter activities
Based on our understanding of molecular mechanism of boron transport in plants, we successfully generated plants that withstand in low and/or high boron conditions. The first trial was to overexpress BOR1. We generated transgenic *A. thaliana* lines expressing BOR1 under the control of CaMV 35S RNA promoter. The transgenic plants showed significant improvement of shoot growth and fertility under limited supply of B (Miwa *et al.* 2006). It was found that the boron loading in xylem was elevated in the transgenic lines and this is likely to be the reason for the enhanced growth under conditions of limited boron supply. It is likely that function of endogenous BOR1 as a xylem loader of B was enhanced in these transgenic lines. The advantage of this approach is that there is no detrimental effect on plant growth under normal or toxic level of B supply, probably due to the degradation of BOR1 under high B conditions (Takano *et al.* 2005).

In the course of the study we noticed that overexpression of B transporter BOR1 improves shoot growth, but not root growth under low B. This is reasonable as BOR1 is a transporter for xylem loading. We examined if overexpression of *NIP5;1* may improve root growth under low B conditions. We first generated transgenic plants overexpressing *NIP5;1*, but the growth of transgenic plants are not improved rather impaired. We then tried not overexpression but enhancement of *NIP5;1* expression by inserting enhancer element upstream of the promoter of *NIP5;1*. The plants with enhanced expression of *NIP5;1* exhibited improved root elongation under low B conditions (Kato *et al.* 2009). Overexpression of *BOR1* in the *NIP5;1* activation tag line resulted in plants with high tolerance to low B.

These represent the first successful improvement of boron deficiency tolerance through modification of transporters. This also reveals potentials of enhancing expression of a mineral nutrient channel gene to improve growth under nutrient limiting conditions. We also succeeded in generating plants with high boron tolerance (Miwa *et al.* 2007). B toxicity is often problem in agricultures in semi arid areas. We constructed transgenic *A. thaliana* lines overexpressing BOR4, a paralog of BOR1. BOR4 accumulation was enhanced when the boron concentration in the media is high. This is a sharp contrast to the case of BOR1. BOR1 diminishes under high B conditions. The transgenic lines overexpressing BOR4 showed remarkable improvement of root and shoot growth under 10 mM B conditions, at this concentration of B the wild type plants fails to grow right after germination. B concentrations in roots and shoots were decreased in these transgenic plant lines. It is likely that overexpressed BOR4 pumps excess boron out of the cell (Miwa *et al.* 2007).

Conclusion

Through the molecular genetic and physiological analysis, boron transporters were identified and their coordinated roles in boron transport from soil to roots and distribution within the plant body are unveiled. By enhancing the activities of appropriate transporters, it is now possible to generate plants tolerant to low or high boron conditions.

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