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## Transgenically enhanced sorbitol synthesis facilitates phloem-boron mobility in rice

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The tolerance of crops to a shortage of boron (B) in the soil varies markedly among species. This variation in tolerance is due, in part, to a species ability to form phloem mobile B-sugar-alcohol complexes (such as B-mannitol or B-sorbitol) which enhance the remobilization of B within the plant. Species lacking the capacity to form B-sugar alcohol complexes are intolerant of even short-term deficits in soil B supply. Here we have genetically engineered rice (*Oryza sativa* L.) cultivar Taipei 309 (TP309) with the sorbitol-6-phosphate dehydrogenase (S6PDH) gene, a key enzyme for sorbitol production, and determined the effect of this transformation on the physi-

ology of B remobilization. Sorbitol was detected in the S6PDH transgenic plants as well as in vector-transformed plants and wild-type (TP 309) plants, although the concentration of sorbitol in the S6PDH transgenic plants was significantly enhanced. Remobilization of B from mature leaves to flag leaves correlated with increased levels of sorbitol. The presence of sorbitol and detection of B remobilization in the wild-type and vector-transformed plants suggests that rice utilizes an unknown pathway for sorbitol synthesis and may partly explain the relative insensitivity of rice to B deficits when compared to other graminaceous crops.

### Introduction

The transport of B in the phloem and the tolerance of crops to B deficits varies greatly among species (Brown and Shelp 1997). The variability in the mobility of B in a species is a consequence of the formation and phloem translocation of B as a B-sugar-alcohol complex, which is highly species specific (Hu et al. 1997).

It has now been demonstrated that introduction of the gene for sorbitol synthesis into a species utilizing molecular techniques can enhance the within-plant nutrient mobility of B and improve plant tolerance to deficiencies (Brown et al. 1999). It was also observed that sorbitol content affects the rate of B uptake and translocation in tobacco (*Nicotiana tabacum* L.) (Bellaloui et al. 1999). The agricultural implications of the role of sugar alcohols in B translocation have been described (Brown et al. 1999). Genetic manipulation of tobacco to produce sorbitol caused significant increase in uptake and

mobility of B (Brown et al. 1999). The increase in B uptake and mobility is expected to contribute to an overall improvement in tolerating low-boron soils and withstanding brief periods of B deficiencies under drought, low transpiration, or rapid plant growth.

The current work further investigates further the role of sugar alcohols in B mobility in rice. Although rice has a low B requirement during vegetative growth, it may have a high B requirement during reproductive growth (Dwivedi et al. 1990), as has been well documented for other crops (Dell and Huang 1997, Blevins and Lukaszewski 1998). Reproductive B deficiency in rice, however, is far less prevalent than the occurrence of B deficiency in other grass crops such as wheat (*Triticum aestivum* L.) or barley (*Hordeum vulgare* L.). Here we determine the physiological basis for the relative tolerance of rice to B deficiency. The effect of overexpression

Abbreviations – S6PDH, sorbitol-6-phosphate dehydrogenase.

of apple S6PDH on the production of sorbitol levels and the mobility of B in transgenic rice were also determined.

## Materials and methods

### Rice transformation

Transgenic plants were developed using a protocol modified from Cheng et al. (1997) and Hiei et al. (1994) as described in Ilag et al. (2000) and as described briefly below.

#### *Initiation of scutellar calli*

Seeds were dehusked and sterilized in 70% ethanol for 1 min and then in 20% commercial sodium hypochlorite for 1 h, washed several times with sterile water, and cultured on callus induction medium (MS basal medium supplemented with 2,4-D 2.0 mg l<sup>-1</sup>, casamino acids 500 mg l<sup>-1</sup>, proline 500 mg l<sup>-1</sup>, sucrose 30 g l<sup>-1</sup> and phytagel 2.5 g l<sup>-1</sup>, pH 5.8) in the dark. The 4-week-old calli derived from mature seed scutellum were used for transformation. Calli were removed from the seeds and divided into small pieces (1–2 mm in diameter) and subcultured for 4–5 days on callus induction medium.

### Bacterial strain and vector construction

The plasmid pBluescript containing the cDNA encoding S6PDH (Kanayama et al. 1992) was digested with *Spe*I and *Eco*RV and ligated into TA cloning vector pCR2.1 (TA Cloning Kit, Invitrogen Corp., Carlsbad, CA, USA) at the *Spe*I and *Eco*RV restriction sites. The apple S6PDH gene (Tao et al. 1995) in the pCR 2.1 vector was then excised with *Bam*HI and *Eco*RV and ligated into the binary vector Ubi-C1301 (Chern et al. 2001) at the *Bam*HI and *Sma*I restriction sites. Ubi-C1301 contains genes for hygromycin resistance and GUS. The GUS gene has an intron in the 5'-end of the coding sequence and is driven by the CaMV 35S promoter. The new construct called Ubi-S6PDH was electroporated into *Agrobacterium* strain EHA 105 and was used for transformation experiments.

For rice transformation, the *Agrobacterium tumefaciens* strain EHA105 carrying ubi-S6PDH was utilized to infect callus induced from mature embryos of cultivar TP 309. Seven independently transformed lines were regenerated and named TR1, TR2, TR3, and TR4, and NC5, or NC7. Two vector control lines were generated and named VT1 and VT2.

### Plant growth

All the plants were grown in a controlled environment facility. The growth chamber condition were as follows: day/night duration: 13.5/11.5 h for the initial 19 weeks, then 11/13 h for the rest of 17 weeks. Temperature 27/22°C (day/night). Relative humidity was 70% constant. Hoagland's solution (Hoagland and Arnon 1950) was

supplied at 1/4 strength for the initial 12 weeks, then supplied at 1/2 strength for 24 more weeks. Silicon was supplied at 1.82 mg l<sup>-1</sup> as Na<sub>2</sub>SiO<sub>3</sub>. Iron was supplied at 1.4 mg l<sup>-1</sup> Fe as ferric-sodium salt of Ethylenediaminetetraacetic acid (EDTA). Boron was added at 0.05 mg l<sup>-1</sup> as boric acid. The nutrient solution was made to pH 6.5 with 0.1 N NaOH. Uniform tillers of each T<sub>0</sub> plant were used as replicates for B translocation studies.

### Southern analysis

Southern analysis was carried out to determine the stable integration of the transgene. Genomic DNA was isolated from young leaf tissue (1 g) of putative transgenic rice plants and untransformed control plants using a protocol modified from Dellaporta et al. (1983). Total DNA (5 µg) was digested with *Sac*I overnight and fractionated on 0.9% (w/v) agarose gel. DNA was transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Inc. Piscataway, NJ, USA) by capillary blotting. The probe, cut by *Spe*I, was a 1.3-kb fragment containing the S6PDH gene (Kanayama et al. 1992) and <sup>32</sup>P-labelled following 'Ready to Go Kit' (Amersham). Pre-hybridization and hybridization were performed according to Amersham's protocol at 65°C for 4 h and 16 h, respectively. Southern blot analysis was repeated three times.

### GUS assay

Expression of GUS in rice plants was assayed as described by Jefferson (1987) with X-Gluc as substrate (5-bromo-4-chloro-3-indolyl glucuronide). The leaf tissues and roots were incubated at 37°C overnight with X-gluc solution in phosphate buffer.

### PCR analysis of the S6PDH transgene in T<sub>1</sub> progeny

T<sub>1</sub> seeds were collected from each T<sub>0</sub> transgenic line. Seeds were dried at 50°C for 2–3 days and then stored at 4°C. The dehusked seeds were hydrated by soaking in distilled water for approximately 12 h. They were transferred onto moist filter paper in Petri dishes and incubated at 30–32°C until germination. Seeds were then transferred to pots containing steam-sterilized Yolo Loam soil and grown in a greenhouse under natural light. Ten seedlings were chosen from the T<sub>0</sub> lines TR1, TR4, NC5, NC7, VT1 and from wild-type TP 309 seedlings. Due to poor germination and seedling establishment, only eight and six plants were available from T<sub>0</sub> lines TR2 and TR3, respectively. DNA was isolated from young leaves using the protocol in Chen and Ronald (1999). PCR amplification of 359 bp of the 3' end of the transgene was accomplished using the primers S6PDH-3 and S6PDH-4 (Tao et al. 1995) in a 25-µl reaction containing 0.5 µM of each primer, 200 µM dNTPs, approximately 50 ng of template DNA, 5 U Taq polymerase in a buffer of 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 0.1% Triton at pH. 9. The thermal cycle was 94°C for 2

min 30 s, then 30 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 1 min, followed by a soak at 4°C. PCR products were separated by gel electrophoresis using a 2% agarose 1× TAE gel and visualized by ethidium bromide staining. PCR was repeated two times and DNA was extracted once.

### Sorbitol analysis

Samples from expanding mature leaves of plants at the vegetative stage were collected and 1 g of frozen tissue was homogenized in a Polytron (Biospec products, Inc., Dremel, WI, USA) with 10 ml deionized cold water. The extract was heated at 100°C for 10 min. After centrifugation, the supernatant was lyophilized. An internal standard, 250 µl solution, which contained 50 µg of xylose, was added to the samples. After the samples were air-dried, 400 µl of acetic anhydride and 60 µl of 1-methyl imidazole were added to acetylate the sorbitol. After 10 min, the reaction was stopped by adding deionized water. The acetylated sugars were partitioned in 2 ml of dichloromethane and dried. Acetylated samples were then dissolved in 100 µl of acetone and analysed using a Perkin-Elmer 8320 gas chromatograph with flame ionization detector. Mass spectrometry was carried out using a mass-selective detector (model 5970, Hewlett-Packard, Palo Alto, CA, USA) to confirm the retention time (Greve and Labavitch 1991, Tao et al. 1995). Four replicates were used for each treatment.

### Boron treatment

At 14 weeks of growth, plants were divided into two groups and subjected to two treatments. Treatment one (control) received 0.05 mg l<sup>-1</sup> B in the medium throughout the entire growth period. Treatment two (foliar B application) received: 0.05 mg l<sup>-1</sup> B in the medium throughout, and as the flag leaf was emerging (14 weeks of growth), 250 mg l<sup>-1</sup> <sup>10</sup>B solution was applied to the two mature leaves immediate below the flag leaf. The <sup>10</sup>B solution was supplied as <sup>10</sup>B-enriched boric acid (99.43% <sup>10</sup>B: 0.57% <sup>11</sup>B) containing 0.05% L-77 (v/v) as surfactant. At the time of <sup>10</sup>B application, the two leaves being fed with <sup>10</sup>B were leaned with the distal portion down and away from all others. The distal portion of the two leaves was briefly immersed in <sup>10</sup>B solution. Care was taken not to contaminate any neighbouring leaves or stems. The two leaves were held in a downward position away from all other leaves until the solution was completely dried. Four <sup>10</sup>B applications were made at a 1 week interval before ear emergence (14th to 17th week), and another three <sup>10</sup>B applications were made during ear emergence (22nd to 24th week). The distal half of the flag leaf was sampled 4 days after the last <sup>10</sup>B application for B analysis.

### <sup>10</sup>B labelling in the tillers

After seed harvest, uniform tillers from the control plants were selected for <sup>10</sup>B labelling as described above for foliar B labelling. Sampling of tillers was made only

twice (due to limited materials). Samples were collected 1 day after <sup>10</sup>B application and 6 days after <sup>10</sup>B application. At sampling the labelled leaves were cut in half longitudinally, so that one half of a single leaf was sampled on day one, and the other half on day six. Control leaves were treated the same way. All leaves were washed in deionized water for 1 min, and then dried in an oven at 70°C for 3 days. Each treatment was replicated three times.

The plants were cultivated for 36 weeks. Seeds from the main stem, once formed were collected from plants at maturity for B analysis. All the samples were ashed at 500°C for 4 h. Boron was analysed by inductively coupled plasma-mass spectrometry (ICP-MS) with Be as internal standard (Brown and Hu 1994).

### Results

Southern blot analysis was carried out to confirm the presence of the S6PDH gene in the T<sub>0</sub> individuals (Fig. 1). Digestion of the T-DNA insert with *Sac*I in those transformants was expected to produce a fragment of the S6PDH gene. Hybridization with the S6PDH probe showed the presence of the expected band in TR1, TR2, TR3, and TR4 (Fig. 1), but not in the wild-type (TP 309), VT1, NC5, or NC7 plants. The lack of the transgene in plants NC5 and NC7, is likely due to escape from selection and these plants were used as regenerated negative controls.

GUS assays revealed GUS expression in TR1, TR2,

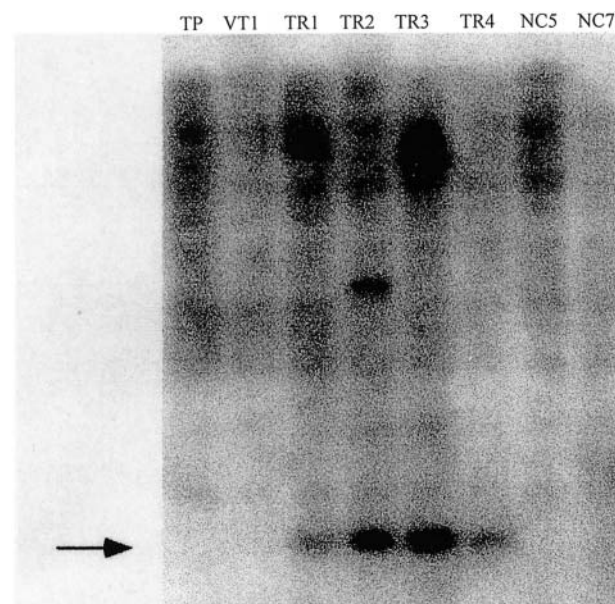


Fig. 1. Southern blot analysis of sorbitol-6-phosphate dehydrogenase (S6PDH) in rice. Hybridization with S6PDH probe demonstrates the presence of the S6PDH gene in transformed plants, TR1, TR2, TR3, and TR4. No S6PDH gene was detected in wild-type Tai-pei 309 (TP), NC5, and NC7, although sorbitol was detected in all plants, including the wild-type. The arrow indicates the position of S6PDH gene band.

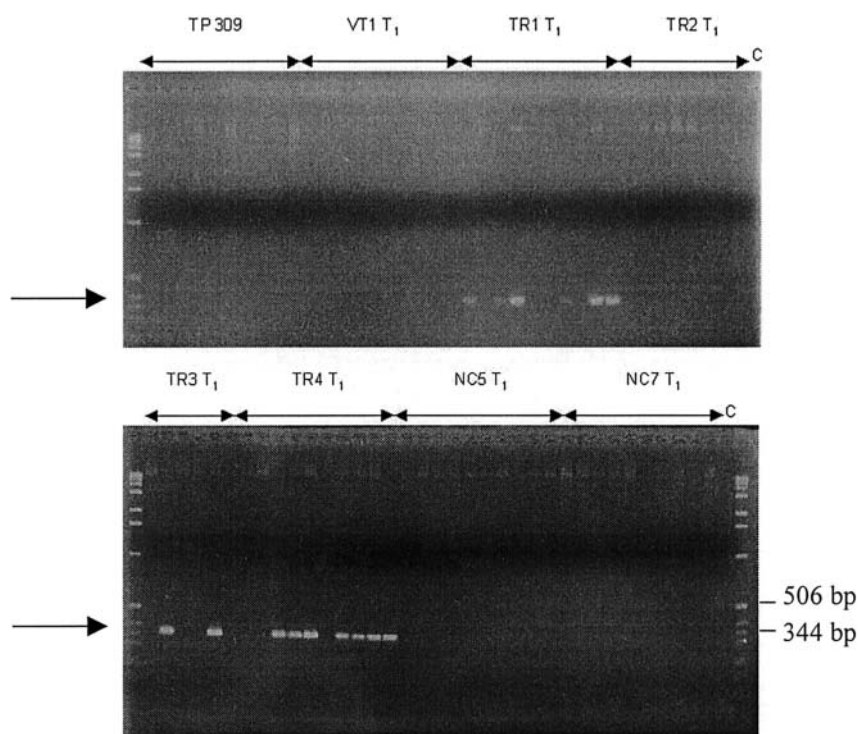


Fig. 2. Segregation of S6PDH transgene in  $T_1$  population in rice. The primers S6PDH-3 and S6PDH-4 were used to amplify a portion of the transgene with the expected size of 359 bp. 'C' is the control reaction with no DNA template. The arrows indicate the position of S6PDH transgene band.

TR3, and TR4 but not in TP309, VT1, NC5, or NC7 (data not shown).

To test for the presence of S6PDH in the  $T_1$  progeny, PCR analysis was carried out on the  $T_1$  individuals. The results are shown in Fig. 2. PCR amplification of the 3' end of the S6PDH transgene resulted in either no product or a single product of the expected size. TP 309,  $T_1$  progeny from the vector-transformed line (VT1), and those lines whose parents were negative for the transgene on the Southern blot (NC5 and NC7) had no PCR product. PCR product segregation ratios of presence of S6PDH:absence were 6:4, 2:4, and 7:3 for TR1  $T_1$ , TR3  $T_1$  and TR4  $T_1$ , respectively. Unexpectedly, none of the  $T_1$  progeny from TR<sub>2</sub> showed a S6PDH PCR product, possibly due to the small number of seeds tested.

Sorbitol concentrations in rice leaves are shown in Table 1. Sorbitol was detected in all plants including the wild-type (TP 309), and the vector transformed plants (VT1 and VT2). The highest concentrations of sorbitol were detected in transgenic (TR) plants, TR1, TR2, TR3, and TR4. Wild-type plants showed the lowest concentrations of sorbitol, followed by VT2 and VT1. Intermediate concentrations were recorded for the negative controls NC5 and NC7 (Table 1).

The ratio of  $^{10}\text{B}/^{11}\text{B}$  in the flag leaf immediately above the mature leaves to which the  $^{10}\text{B}$  was applied, is shown in Table 1. As flag leaves did not receive foliar B application, any increase in the mole  $^{10}\text{B}/^{11}\text{B}$  ratio reflects retranslocation of B from mature leaves. Wild-type and vector-transformed plants showed a significant increase in

Table 1. Mole ratio of  $^{10}\text{B}/^{11}\text{B}$  in the emerging flag leaves and sorbitol concentration in mature leaves of rice plants after sorbitol synthesis gene insertion. Mole ratio of  $^{10}\text{B}/^{11}\text{B}$  was analysed in the emerging flag leaves after  $^{10}\text{B}$ -enriched boric acid application to the mature leaves. Percentage (%) increase was compared with control plants (TP 309).

Plant <sup>1</sup>	$^{10}\text{B}/^{11}\text{B}$ (%)	$^{10}\text{B}/^{11}\text{B}$ increase ( $\mu\text{mol g}^{-1}$ FW)	Sorbitol (%)	Sorbitol increase
TP 309	0.37 c <sup>2</sup>	Control	0.89 d <sup>2</sup>	Control
VT1	0.36 c	-4	1.45 c	63
VT2	0.33 c	-12	1.22 cd	37
TR2	0.47 bc	26	5.22 a	487
TR3	0.63 b	69	5.23 a	488
TR4	1.13 a	202	5.26 a	491
NC5	No data	No data	2.44 b	174
NC7	No data	No data	2.42 b	172

<sup>1</sup>TP 309 is the wild-type Tai-pei 309, VT1 and VT2 were transformed with only the vector ubi-C1301, while TR2, TR3, and TR4 were transformed with ubi-S6PDH.

<sup>2</sup>Shown are means of three replicates for  $^{10}\text{B}/^{11}\text{B}$  ratio and four replicates for sorbitol; mean separation was performed using Tukey-Kramer multiple comparisons test. Numbers within a column bearing the same letters are not significantly different ( $P < 0.05$ ).



the mole  $^{10}\text{B}/^{11}\text{B}$  ratio. The largest increase in  $^{10}\text{B}/^{11}\text{B}$  ratios was found in the S6PDH transgenic plants TR3 and TR4.

The remobilization of B from mature leaves of the tillers after foliar application was evident in all  $T_0$  lines tested (TP309, VT1, VT2, TR3, TR4, and NC7) but was not consistently correlated to sorbitol concentration or presence of the transgene in all plants (data not shown). Except for the wild-type, seeds from the main stem of the plants tested showed an enrichment of  $^{10}\text{B}$  as reflected by the increase in  $^{10}\text{B}/^{11}\text{B}$  ratio (data not shown).  $^{10}\text{B}$  enrichment was much lower in seeds than in flag leaves, which is a consequence of the long developmental period for the seeds and the resultant dilution of  $^{10}\text{B}$  by  $^{11}\text{B}$  derived from the root uptake. Significant enrichment of the seed with B was not well correlated with sorbitol levels.

## Discussion

The decrease in the  $^{10}\text{B}/^{11}\text{B}$  ratio in mature leaves of all  $^{10}\text{B}$ -treated plants occurs as the result of two processes: the re-translocation of foliar-applied  $^{10}\text{B}$  and the dilution with natural abundance (20%  $^{10}\text{B}$ : 80%  $^{11}\text{B}$ ) obtained from the roots. Calculations of total B uptake during this period demonstrated that isotope dilution accounts for less than half of the decrease in  $^{10}\text{B}/^{11}\text{B}$  ratio over this period (data not shown). The decrease in  $^{10}\text{B}/^{11}\text{B}$  ratio was therefore mainly due to the re-translocation of the  $^{10}\text{B}$  label from the treated leaves. In agreement with this conclusion the significant  $^{10}\text{B}$  enrichment in the untreated young leaves (flag leaves) that was detected in all plants could only have occurred as a result of B re-translocation from treated leaves. Further evidence of  $^{10}\text{B}$  movement out of the treated leaves was found in the significant enrichment of  $^{10}\text{B}$  in the untreated seeds in all plants except the wild-type (data not shown). The reason for the lack of enrichment in the seeds of wild-type may be partially due to the dilution effect of the many seeds produced from this high yielding line. Taken together, the results suggest that foliar-applied  $^{10}\text{B}$  was re-translocated from the mature leaves of transgenic and non-transgenic rice to young untreated leaves or seed organs. Boron is phloem-mobile in all the rice plants used in this experiment.

Sugar analysis indicated the presence of sorbitol in all plants, including the wild-type. The results from both sugar analysis and re-translocation studies support the hypothesis that the presence of sorbitol results in the movement of foliar-applied  $^{10}\text{B}$  in all plants. The rate at which foliar-applied  $^{10}\text{B}$  was translocated from mature treated leaves and appeared in the young leaves varied with sorbitol concentration. The highest increase in  $^{10}\text{B}/^{11}\text{B}$  ratio of flag leaf after  $^{10}\text{B}$  labelling was observed in TR3 and TR4, which also exhibited the highest concentrations of sorbitol. That sorbitol can enhance phloem B mobility is in agreement with previous results, which indicate that in all sorbitol-producing species, boron is highly mobile (Brown and Hu 1996, 1998, Hu et al.

1997). In addition, boron becomes phloem-mobile in tobacco, when sorbitol-producing gene was introduced (Brown et al. 1999, Bellaloui et al. 1999).

The detection of sorbitol in wild-type, VT1, and VT2 was unexpected as we have found no previous report of its presence in rice. Further, the production of sorbitol in non-transformed rice is clearly not the result of the presence of the S6PDH transgene as no homologous gene was detected by Southern blot analysis. However, this does not preclude the presence of a native variant of the S6PDH gene in rice. The presence of the S6PDH transgene gene in rice, however, further enhanced sorbitol production above the basal level observed in the wild-type. These results suggest that a biochemical pathway exists for sorbitol production in rice. Enzymes known to be involved in sorbitol metabolism include S6PDH, NAD-dependent sorbitol dehydrogenase, NADP-dependent sorbitol dehydrogenase, and sorbitol oxidase (Loescher et al. 1982, Yamaki 1984). The activity of these enzymes in rice has not been previously assayed.

The current results suggest that genetic manipulation of rice can increase the concentrations of sorbitol. The presence of sorbitol, irrespective of its source of synthesis, however, clearly facilitates B mobility and may contribute to the tolerance of rice to B deficiency.

The mole ratio of  $^{10}\text{B}/^{11}\text{B}$  in the flag leaves was not tested in the negative control plants NC5 and NC7, yet in the seed they showed significant enrichment for  $^{10}\text{B}$  as compared to the TP 309, VT2, but not TR1 or TR4. This may be due to the reduced seed production observed in NC5 and NC7, a dilution effect, or some other factors (data not shown).

Future work is required to confirm that the transgene co-segregates with increased sorbitol production and enhanced boron mobility in the transgenic progeny ( $T_1$  families). Also, further studies are required to determine the existence of an alternative biochemical pathway for sorbitol production in rice. As an initial step, enzyme activities involved in the sorbitol production other than S6PDH can be analysed in the wild-type rice. Once a pathway is identified, antisense technology may be used to block sorbitol production. In this way a causal relationship between sorbitol production and B re-translocation can be established.

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