Chapter 6

Differential regulation of root architecture in autotetraploid *Arabidopsis thaliana* under boron deficiency

Abbreviations

B, boron; PCR, polymerase chain reaction

Introduction

Boron transporters *BOR1* and *NIP5;1* accelerate boron (B) uptake from media and support growth under B deficiency in *Arabidopsis thaliana* (Takano et al. 2002, Takano et al. 2006). Recent studies in our laboratory also showed improvement of plant tolerance to B deficiency through overexpression of *BOR1* or activation of *NIP5;1* (Miwa et al. 2006, Kato et al. not published yet). The first example of attached low-B tolerance was found in transgenic tobacco expressing *sorbitol-6-phosphate dehydrogenase* (*S6PDH*) gene cloned from apple (Brown et al. 1999). Although many additional genes to *NIP5;1* whose transcript accumulations are upregulated by B deficiency are identified in transcriptome analyses in *Arabidopsis*, no gene to improve low-B tolerance is identified from these analyses (Kasajima and Fujiwara 2007; chapter 4). To identify novel genes which improve tolerance to B deficiency, *Arabidopsis* activation-tagged lines were screened under B deficiency and three candidate tolerant lines were isolated, which were designated as A119B, A142B, and A152B (chapter 5). These mutants were characterized in this chapter. From the analysis of A152B, effect of polyploidy on morphology under low-B was found.

Results

Differential growth of A119B line

Growth of three activation-tagged lines designated A119B, A142B, and A152B were observed under B deficiency. Growth of A119B was observed under normal and low-B conditions (Fig.

6-1A,B,C). Although there was no obvious difference in morphology of A119B from wild-type, this mutant tends to be bigger than wild-type even under normal condition (Fig. 6-1A). Difference in growth is obvious under low-B conditions (Fig. 6-1B,C). Because all plants are bigger than wild-type, S_2 progeny of A119B is homozygous for its mutation. Adaptor polymerase chain reaction (adaptor PCR) analysis identified a T-DNA insertion site near *At1g34250* and *At1g34260* on the chromosome I. T-DNA was inserted in homozygous in every analyzed eight S_2 plants. Linkage analysis to reveal linkage between this T-DNA insertion and growth phenotype failed to judge the correlation because growth phenotype was not clear in backcrossed F_2 progeny.

Differential growth of A142B line

Growth of A142B was observed under a low-B condition (Fig. 6-1D,E). In this analysis wild-type and S_2 progeny of A142B were grown on media containing 0.2 μM borate for ten days. Root elongation of wild-type is greatly inhibited on this medium (Fig. 6-1D). On the other hand, Root elongation of a part of A142B plants are not inhibited or inhibited to a much smaller extent on the same medium. Phenotype is segregating in S_2 progeny. No homozygous S_3 segregant is isolated at present and the segregation ratio of this mutation is not clear. Although T-DNA was detected by PCR, no genomic franking sequence to T-DNA was identified after adaptor PCR.

Differential growth of A152B line

Growth of A152B was also observed under normal and low-B conditions (Fig. 6-2A,B,C). Under normal condition, A152B was slightly bigger than wild-type (Fig. 6-2A). Under low-B conditions, root elongation of A152B was clearly improved compared with that of wild-type (Fig. 6-2B,C). This growth phenotype was segregating in S_2 progeny between wild-type growth and long-root growth. All of S_3 progeny derived from wild-type S_2 grew like wild-type and all of S_3 progeny derived from long-root S_2 grew like long-root mutant. Growth of one of the long-root segregants (segregant #3) is shown in the figure. The segregation pattern observed in A152B is not consistent with phenotypes caused by T-DNA insertions. A franking sequence to T-DNA was detected in *At3g15410* genetic region on the chromosome III. Because this insertion was detected in homozygous in all eight S_2 plants analyzed, there is no linkage between this T-DNA insertion and long-root phenotype.

A152B is tetraploid

Because morphology of A152B such as bigger seeds, bigger flowers, and bigger pollens were similar to tetraploid, cell ploidy of A152B was measured in two-week whole shoots (Fig. 6-2D,E). Nuclei were stained with DAPI and fluorescence was quantified with flow-cytometry. Frequency distributions were compared between diploid wild-type and long-root segregant of A152B. In diploid wild-type, peaks for nuclei containing two sets (2C), four sets (4C), and the following doubling numbers of chromosomes are detected (Fig. 6-2D). Two sets of chromosomes are originally contained in cells of diploid plants, and then duplication of nuclear DNA is caused

by both endoreduplication and arrest of cell cycle at G_2 phase (Sugimoto-Shirasu and Roberts 2003). In long-root segregant of A152B, the peak for nuclei containing two sets of chromosomes (2C) is lacking and peaks for nuclei containing four sets (4C), eight sets (8C), and the following doubling numbers of chromosomes are detected (Fig. 6-2E). This means long-root segregant of A152B is tetraploid. When ploidy was observed in S_2 plants, wild-type segregants were diploids and long-root segregants were tetraploids.

Growth of tetraploid under B deficiency

To know if long-root phenotype under low-B is a common feature of autotetraploid *Arabidopsis*, two additional autotetraploid lines were obtained from Arabidopsis Biological Resource Center (ABRC). CS3900 is a spontaneous tetraploid obtained during tissue culture (Wang et al. 2004). CS3151 is also a tetraploid according to annotation in TAIR web-site (http://www.arabidopsis.org). Ploidies of obtained seeds were observed and these were actually tetraploids. These tetraploids along with corresponding wild-type diploids were grown on normal or low-B media (Fig. 6-3). There was no obvious difference in growth between diploid and tetraploid on normal medium (Fig. 6-3A). On the other hand, there was clear difference between diploid and tetraploid on low-B media (Fig. 6-3B,C). Root elongation of tetraploid was improved under B deficiency.

Biomass of polyploids

Fresh weights were measured in diploid, triploid, and tetraploid plants under normal and low-B conditions (Fig. 6-4A,B,C). Triploids were prepared by crossing diploid wild-type and tetraploid segregant of A152B. Diploid stamen was crossed to tetraploid pistil (designated as p4s2) or vice versa (p2s4). Although p4s2 triploid seeds were set normally, p2s4 triploid seeds were set only a few in every silique and no p2s4 seed germinated on plates. On a normal medium, p4s2 triploids and tetraploids were somewhat bigger than diploid and the main roots of p4s2 triploids and tetraploids were also somewhat longer than those of diploids (Fig. 6-4A). On the other hand on a low-B medium, main roots of p4s2 triploids and tetraploids were clearly longer than those of diploids (Fig. 6-4B). Whole shoots and whole roots of these plants were weighted (Fig. 6-4C). On the normal medium both whole shoot and whole root are bigger in polyploids than in diploids. Although both whole shoot and whole root are also bigger in polyploids than in diploids, the proportions between polyploids and diploids are not largely different between normal and low-B conditions.

Epidermal cells were also observed. Two factors can improve root elongation. The first factor is improved cell devision which cause higher number of cells and the second factor is improved cell elongation. Diploids wild-type and tetraploid were grown for one week on normal and low-B media, and epidermal cells in the root-hair zone near the root tip were observed by laser-scanning fluorescent confocal microscopy (Fig. 6-4D,E). In diploid, cell elongation is inhibited by B deficiency and frequency of root hairs became condensed. In tetraplid, this inhibition of cell elongation by B deficiency is milder and cells are clearly longer in tetraploid

than in diploid under low-B.

Growth of tetraploid under various stress conditions

To assess the significance of polyploidization in nature, growth of diploid and tetraploid was also observed under various stress conditions (Fig. 6-5). Diploid wild-type (ecotype Col-7) and tetraploid A152B were grown under low-nitrogen, low-phosphorus, low-sulfur, high-boron, high-salt, and low-potassium conditions as well as under a normal condition. Although tetraploid tended to be bigger than diploid in all growths, tetraploid did not seem tolerant to any stress. Because tetraploid especially looked bigger than diploid under high-B and high-salt conditions, the ratio in fresh weights of whole shoots between diploid and tetraploid was compared. In this comparison, no large difference in ratio was observed between normal and stress conditions, suggesting that tetraploid is not tolerant to any stresses observed here.

Discussion

In this chapter, three activation-tagged lines which show improved root elongation under B deficiency were analyzed. Although mutations in A119B and A142B that cause observed phenotypes are not identified yet, these lines indicate novel mechanisms for tolerance to B deficiency or otherwise differential regulation of root architecture under B deficiency.

The third line A152B was tetraploid. Because S_2 progeny of this line was segregating between diploid and tetraploid, it is deduced that tetraploid cells were induced in S_1 plant. In other words, the S_1 plant was a ploidy chimera. Because long-root phenotype was also observed in other autotetraploid lines, a new phenotype of autotetraploid *Arabidopsis* was identified in this study. Then, what is the significance of longer root in tetraploid under B deficiency? Can we describe this as tolerance of tetraploid to B deficiency? When biomass was measured, both shoot and root of tetraploid was clearly heavier than those of diploid under normal condition. Although both shoot and root tended to be heavier in tetraploid also under low-B condition, the ratio between diploid and tetraploid was not largely different between normal and low-B conditions. Thus, the long-root phenotype of tetraploid under B deficiency does not represent tolerance to B deficiency, but it represents differential regulation of root architecture in tetraploid under B deficiency, which includes improved root-cell elongation under B deficiency. Because infertility is one of the important disorders caused by B deficiency, sterility under B deficiency was also compared between diploid and tetraploid. In this observation, no difference in fertility was observed between diploid and tetraploid, further confirming that there is no difference in tolerance to B deficiency between diploid and tetraploid *Arabidopsis*. When cell ploidy was compared between normal and low-B conditions in wild-type diploid, its proportion was slightly different only in root. This difference in cell ploidy may cause some difference in root architecture under B deficiency in *Arabidopsis*.

As polyploid species are found at areas of higher altitudes and more polar latitudes than their ancestral diploids in nature, it is hypothesized that polyploids are more tolerant to some environmental stresses. The estimation that polyploidization is an ongoing process in plant species also support this idea (Otto and Whitton 2000). To assess this possibility in *Arabidopsis*, diploid and tetraploid plants were cultivated under various stress conditions in the present study. Although tetraploid plants were bigger than diploid plants, they were not tolerant to nitorgen deficiency, phosphorus deficiency, potassium deficiency, sulfur deficiency, boron toxicity, and salt toxicity. Tolerance of polyploid may be restricted to some specific stresses which was not analyzed in this study such as drought or cold. Or otherwise, tolerance may be restricted to some plant species and polyploidization is accelerated only in such species. Most wild *Arabidopsis* lines are diploid, although there are a few tetraploid *Arabidopsis* lines including ecotype Wa-1. There are several species which are heavily polyploidized (Leitch et al. 2005). Observing tolerance of these species to environmental stresses may provide insights into its preferential polyploidizatoin.

Materials and methods

Plant materials

Activation-tagged lines A119B, A142B, A152B are derived from CS21991 pool of *Arabidopsis thaliana* (L.) Heynh. ecotype Col-7. Tetraploid lines CS3900 and CS3151 obtained from Arabidopsis Biological Resource Center and diploid wild-types ecotype Col-1, Col-7, Landsberg *erecta* (L*er*) were also used.

Plant culture conditions

Surface-sterilized seeds were sown on sterilized MGRL growth media (Fujiwara et al. 1992) containing 1% (w/v) sucrose. For preparation of sulfur-deficient media, agar (Wako, Osaka, Japan) was rinsed with ion exchange water several times to remove contained sulfate. Sulfate added to solution was also substituted by chloride to reduce sulfate concentration (Hirai et al. 1995). For preparation of boron-deficient media, borate added to media was reduced and media were solidified with 0.5% (w/v) gellangum (Wako, Osaka, Japan). Borate concentration was increased to prepare boron-excessive media. For preparation of nitrogen-deficient media, nitrate was substituted by equimolar amount of chloride. Media were solidified with 1% (w/v) agar. For preparation of phosphorus-deficient media, phosphate was reduced. Media were bufferized with 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.8) and solidified with 1% agar. For preparation of potassium-deficient media, potassium ion was substituted by half-equimolar amount of calcium ion. Media were solidified with 1% agar. For preparation of high-salt media, sodium chloride was added to standard salt and solidified with 1% agar. The seeds were incubated on culture plates at 4°C for 4 days and were then grown at 22°C under

fluorescent lamps with a 16-h light/8-h dark cycle.

PCR detection of T-DNA and adaptor PCR

T-DNA insertion was detected by PCR with primer set ZE-FR (5'-TTG TCG TGA ACG GTG AGA AG-3' / 5'-CGA GTC AGT GAG CGA GGA A-3') which amplifies a 411-bp region of pSKI015 (Weigel et al. 2000).

Method for adaptor PCR was partially modified from previous methods (Siebert et al. 1995, Yamamoto et al. 2003, Ichikawa et al. 2003). To prepare adaptor-ligated DNA, 2.5 µg of genomic DNA was digested in 100 µL volume with 8 U each of *Bgl*II, *Xho*I, and *Eco*RI overnight at 37°C using H buffer supplied by manufacturer (Takara, Kyoto, Japan). The DNA was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) vol/vol, once with chloroform, and then precipitated by addition of 1/10th volume of 3 M sodium acetate and 2 volume of ethanol. After vortex, the tubes were immediately centrifuged at 14,000 rpm in a microcentrifuge for 10 minutes. The pellets were washed with 70% ethanol and immediately centrifuged as above for 5 minutes, air dried and dissolved in 20 µL of sterilized water. Ten µL of DNA was then ligated to adaptor mixture (5'-GTA ATA CGA CTC ACT ATA GGG CAC GCG TGG TCG ACG GCC CGG GCT GGT-3', 5'-AAT TAC CAG CCC-AmC7-3', 5'-GAT CAC CAG CCC-AmC7-3', 5'-TCG AAC CAG CCC-AmC7-3') overnight in 20 µL volume at 16°C with T4 DNA ligase (Takara, Kyoto, Japan). The ligation reaction was terminated by incubation of the tubes at 70°C for 5 minutes, then diluted 50-fold by addition of sterilized water. The first PCR was performed with primers AP1 (5'-GTA ATA CGA CTD ACT ATA GGG C-3') and 015A (5'-CTC ATC TAA GCC CCC ATT TG-3'), for 26 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 6 minutes, followed by 72°C for 3 minutes in 50 µL volume with Ex Taq (Takara, Kyoto, Japan). The PCR product was diluted 100-fold by addition of sterilized water. The second nested PCR was performed with primers AP2 (5'-ACT ATA GGG CAC GCG TGG T-3') and 015B (5'-CCC ATT TGG ACG TGA ATG TAG-3') under the same conditions as the first PCR. PCR product was purified after electrophoresis and sequenced with primer 015B or 015C (5'-CGT GAA TGT AGA CAC GTC GAA-3').

Co-dominant T-DNA specific primers to detect T-DNA insertions were designed. 119F (5'-GGA ATT TCT TTA CTG GGA TCT TTG-3') and 119R (5'-AGA TGT TTG GCT GAT AAC ATC AT-3') amplifies a 1,044-bp genomic DNA in wild-type but not in A119B line. Instead 119F and 015B amplifies a 487-bp fragment at T-DNA junction in A119B. 152F (5'-CAA CAT TTC AAG TCT TCC TCC A-3') and 152R (5'-CGT ACT TCT TTT GTC CCT TGA TG-3') amplifies a 961-bp genomic fragment, and 152F and 015B amplifies a 499-bp fragment at T-DNA junction in A152B.

Cell ploidy analysis

Cell ploidy was measure as described (Yoshizumi et al. 2006), as follows. Nuclei were extracted by chopping tissues with a few drops of chopping buffer and stained with CyStain UV precise P (Partec) following the manufacturer's protocol. Flow cytometric analysis was performed by a Ploidy Analyzer (Partec).

Measurement of root-cell length

Roots were stained with propidium iodide and cell lengths were observed by confocal laser scanning microscopy (Leica TCS-SP).

References

Brown PH, Bellaloui N, Hu H, Dandekar A (1999) Transgenically enhanced sorbitol synthesis facilitates phloem boron transport and increases tolerance of tobacco to boron deficiency. Plant Physiol 199: 17-20

Fujiwara T, Hirai MY, Chino M, Komeda Y, Naito S (1992) Effects of sulfur nutrition of expression of the soybean seed storage protein genes in transgenic petunia. Plant Physiol 99, 263-268

Hirai MY, Fujiwara T, Chino M, Naito S (1995) Effects of sulfate concentrations on the expression of a soybean seed storage protein gene and its reversibility in transgenic *Arabidopsis thaliana*. Plant Cell Physiol 36: 1331-1339

Ichikawa T, Nakazawa M, Kawashima M, Muto S, Gohda K, Suzuki K, Ishikawa A, Kobayashi H, Yoshizumi T, Tsumoto Y, Tsuhara Y, Iizumi H, Goto Y, Matsui M (2003) Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T1 generation. Plant J 36: 421-429

Kasajima I, Fujiwara T (2007) Identification of novel *Arabidopsis thaliana* genes which are induced by high levels of boron. Plant Biotech 24: 355-360

Leitch IJ, Soltis DE, Soltis PS, Bennett MD (2005) Evolution of DNA amounts across land plants (Embryophyta). Ann Bot 95: 207-217

Miwa K, Takano J, Fujiwara T (2006) Improvement of seed yields under boron-limiting conditions through overexpression of BOR1, a boron transporter for xylem loading, in *Arabidopsis thaliana*. Plant J 46: 1084-1091

Otto SP, Whitton J (2000) Polyploid incidence and evolution. Annu Rev Genet 34: 401-437

Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. Nucl Acid Res 23: 1087-1088

Sugimoto-Shirasu K, Roberts K (2003) "Big it up": endoreduplication and cell-size control in plants. Curr Opin Plant Biol 6: 544-553

Takano J, Noguchi K, Yasumori M, Kobayashi M, Gajdos Z, Miwa K, Hayashi H, Yoneyama T, Fujiwara T (2002) Arabidopsis boron transporter for xylem loading. Nature 420: 337-340

Takano J, Wada M, Ludewig U, Schaaf G, von Wirén N, Fujiwara T (2006) The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. Plant Cell 18: 1498-1509

Wang J, Tian L, Madlung A, Lee HS, Chen M, Lee JJ, Watson B, Kagochi T, Comai L, Chen ZJ (2004) Stochastic and epigenetic changes of gene expression in Arabidopsis polyploids. Genetics 167: 1961-1973

Weigel D, Ahn JH, Blázquez MA, Borovitz JO, Christensen SK, Frankhauser C, Ferrándiz C, Kardailsky I, Malancharovil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in Arabidopsis. Plant Physiol 122: 1003-1013

Yamamoto YY, Tsuhara Y, Gohda K, Suzuki K, Matsui M (2003) Gene trapping of the *Arabidopsis* genome with a firefly luciferase reporter. Plant J 35: 273-283

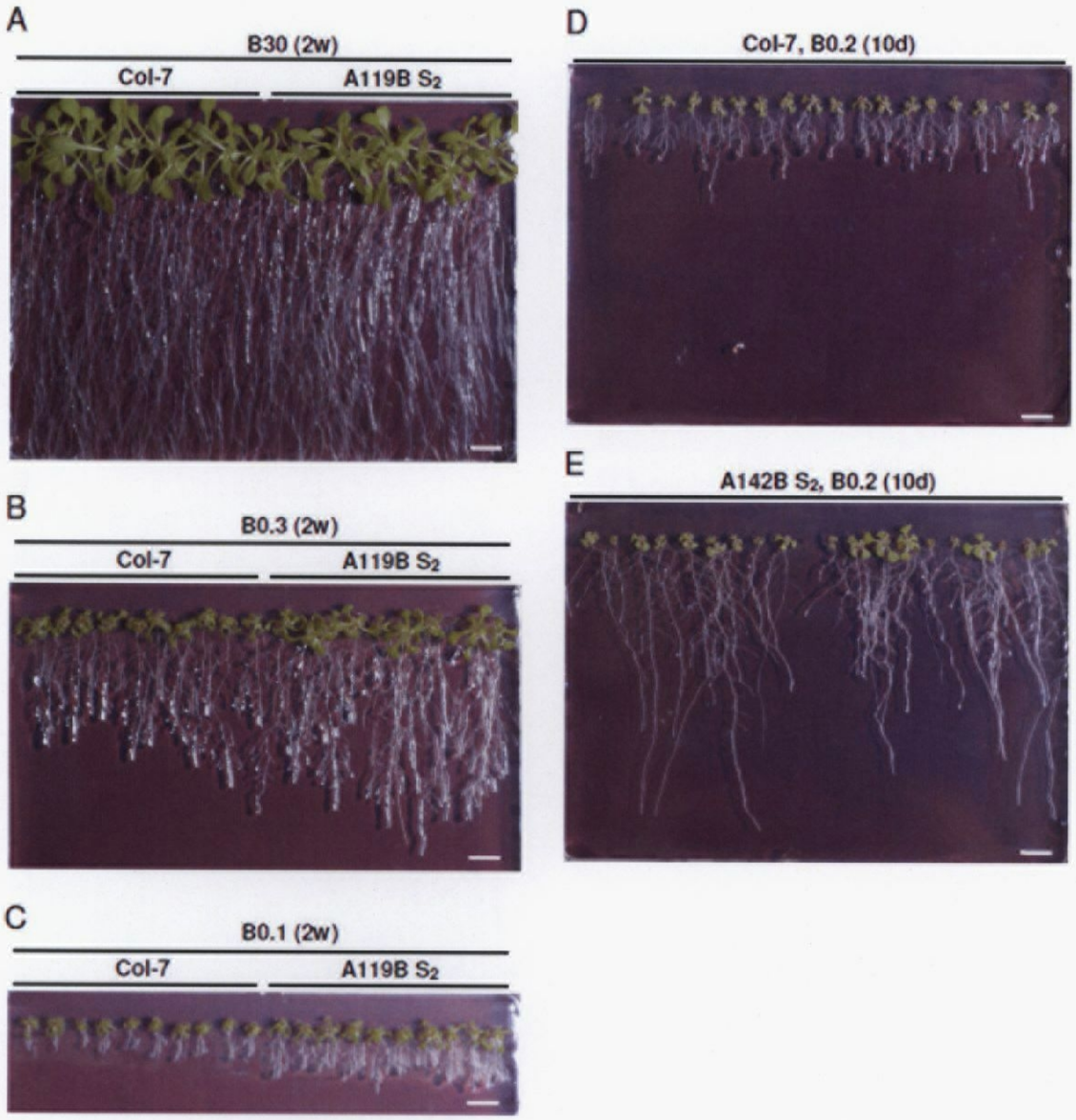

Fig. 6-1. Growth of A119B and A142B. S_2 progenies of A119B and A142B together with wild-type (Col-7) were grown under normal conditions or low-B conditions. (A) Wild-type and A119B plants were grown for two weeks on normal medium containing 30 μM borate. (B) Wild-type and A119B were grown for two weeks on low-B medium containing 0.3 μM borate. (C) Wild-type and A119B were grown for two weeks on low-B medium containing 0.1 μM borate. (D) Wild-type plants were grown for ten days on low-B medium containing 0.2 μM borate. (E) A142B plants were grown for ten days on low-B medium containing 0.2 μM borate. Bars = 1 cm.

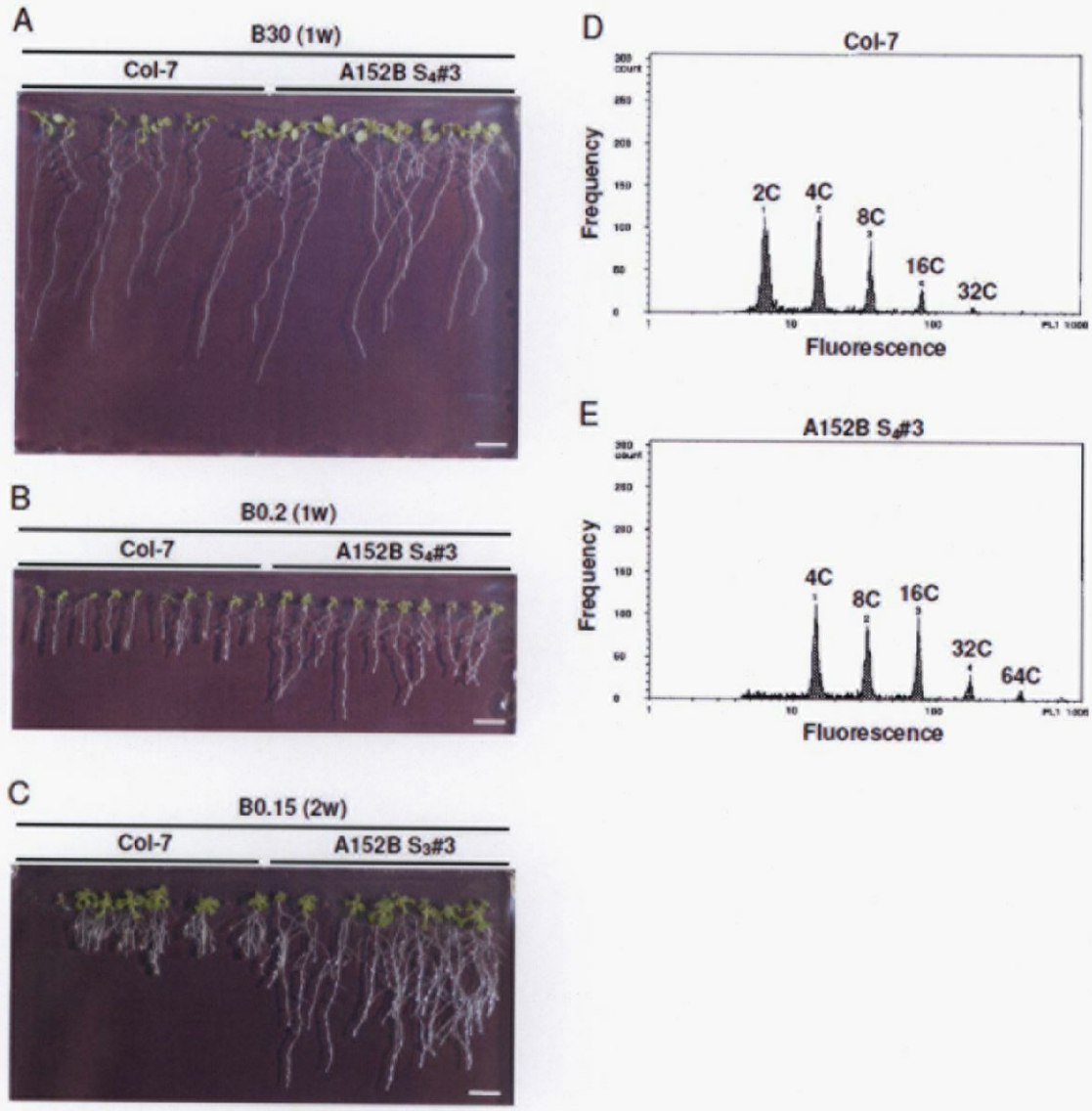

Fig. 6-2. Properties of A152B. Wild-type (Col-7) and long-root segregant (segregant #3) A152B plants were grown under normal condition or low-B conditions (A, B, C). (A) Wild-type and A152B were grown for one week on normal medium containing 30 μM borate. (B) Wild-type and A152B were grown for one week on a low-B medium containing 0.2 μM borate. (C) Wild-type and A152B were grown for two weeks on a low-B medium containing 0.15 μM borate. Bars = 1 cm. Cell ploidy was measured in whole shoots of two-week plants (D, E). (D) Cell ploidy of wild-type (Col-7). (E) Cell ploidy of long-root segregant. In total 3,000 cells were measured.

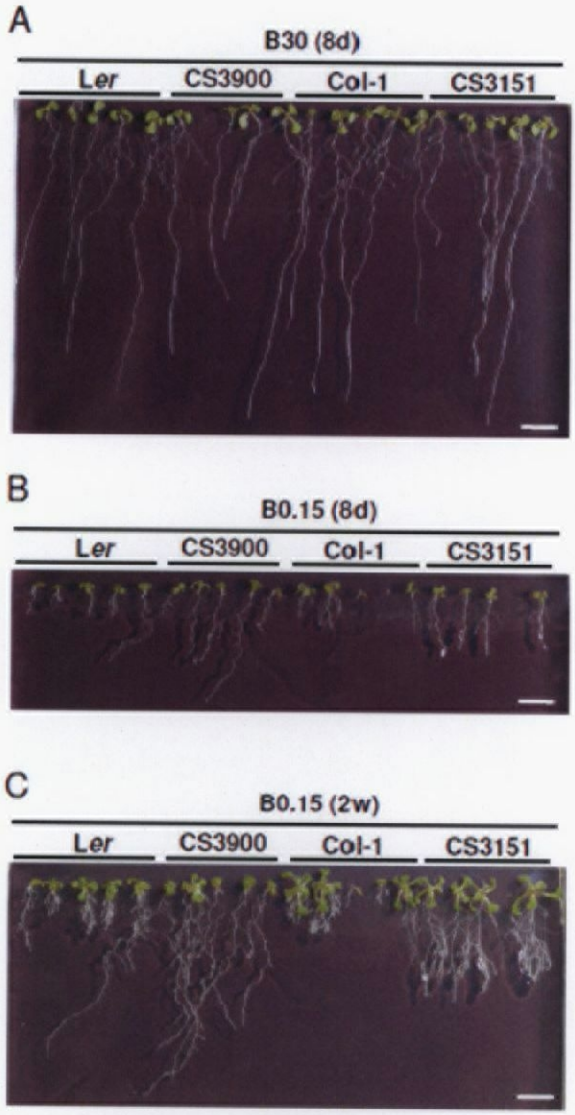

Fig. 6-3. Growth of tetraploids. Tetraploids obtained from stock center (CS3900, CS3151) and their corresponding diploid wild-types (L*er*, Col-1) were grown on normal or low-B media. (A) Plants were grown on a normal medium containing 30 μM borate for eight days. (B) Plants were grown on a low-B medium containing 0.15 μM borate for eight days. (C) Plants were grown on a low-B medium containing 0.15 μM borate for two weeks. Bars = 1 cm.

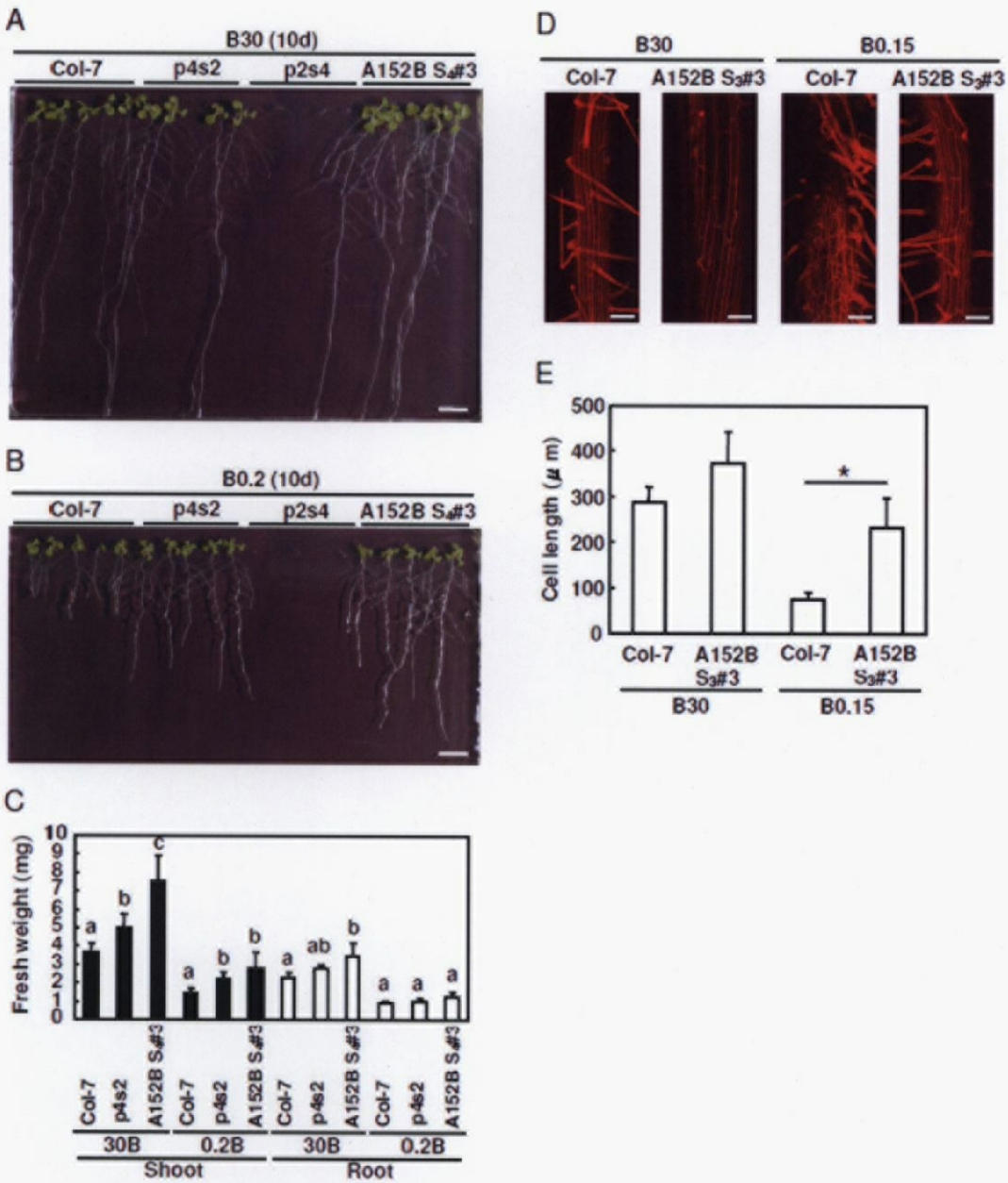

Fig. 6-4. Growth of tetraploids and triploids. Diploid wild-type (Col-7), triploids (p4s2, p2s4) and tetraploid (A152B) were grown for ten days (A, B, C). Triploid seeds were prepared by crossing diploid stamen to tetraploid pistil (p4s2) or vice versa (p2s4). p2s4 seeds were not fertile. (A) Plants were grown on a normal medium containing 30 μM borate. Bar = 1 cm. (B) Plants were grown on a low-B medium containing 0.2 μM borate. Bar = 1 cm. (C) Fresh weights of whole shoots and whole roots grown as shown in (A) and (B). n = 4. Different alphabets (a, b, c) represent statistical difference between data ($P < 0.05$, a < b < c). (D) Epidermal cells were observed in root-hair zones near the root tips of one-week plants. Plants were grown on normal (B30) or low-B (B0.15) media. (E) Measurement of cell lengths of the same cells as (D). Asterisk indicates statistical difference ($P < 0.05$). n = 4. Bar = 100 μm.

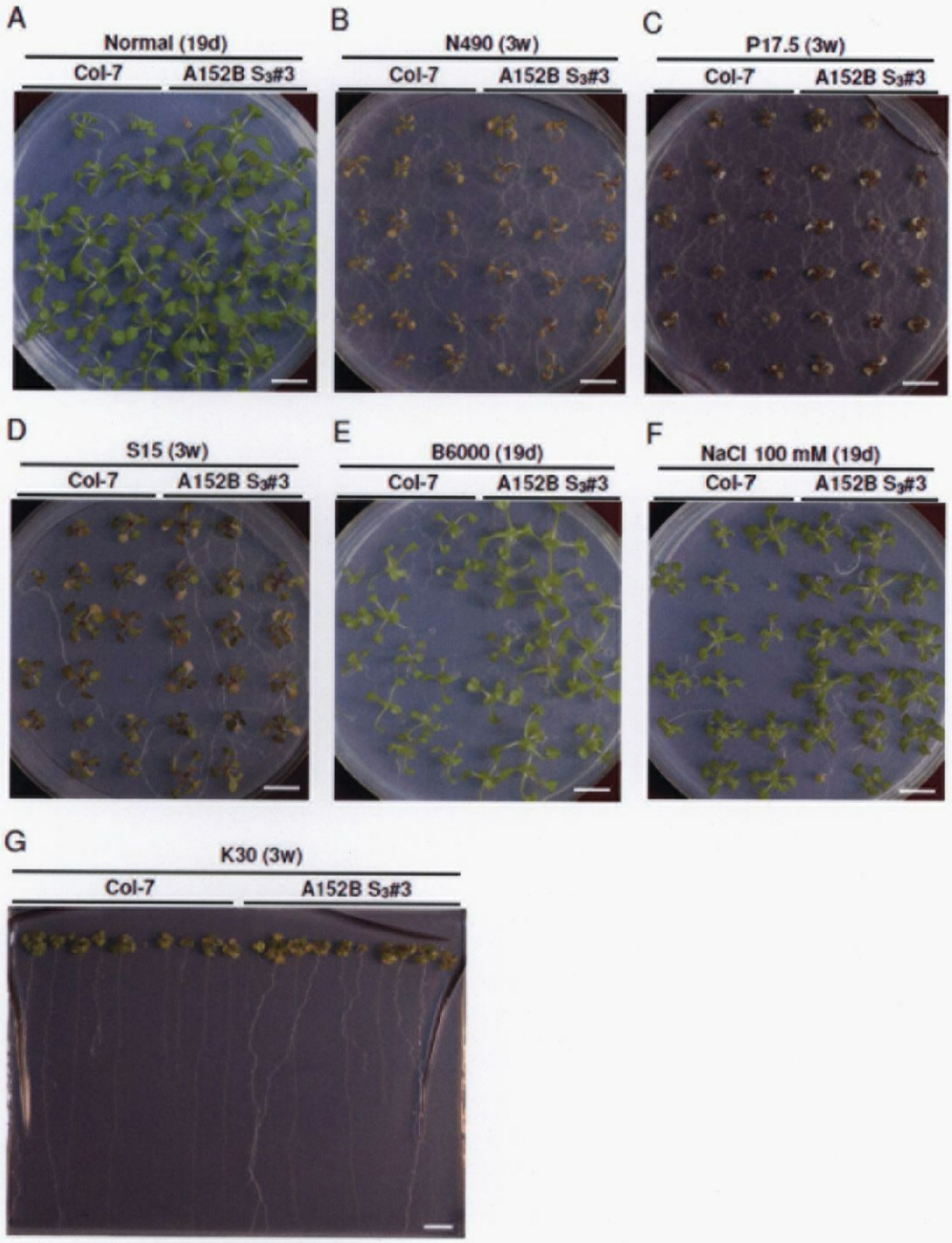

Fig. 6-5. Growth of tetraploid under various conditions. Diploid wild-type (Col-7) and tetraploid (A152B) were grown for 19 days or three weeks on various media. (A) Normal medium for control. (B) Low-nitrogen medium containing 490 μM nitrate. (C) Low-phosphorus medium containing 17.5 μM phosphate. (D) Low-sulfur medium containing 15 μM sulfate. (E) High-B medium containing 6,000 μM borate. (F) High-salt medium containing 100 mM sodium chloride. (G) Low-potassium medium containing 30 μM potassium ion. Bars = 1 cm.

Acknowledgements

Tetraploid lines CS3900 and CS3151 were provided by Arabidopsis Biological Resource Center, Prof. Luca Comai, and Prof. George Redei. Precious instructions were made by Prof. Luca Comai and Dr. Maruthachalam Ravi for ploidy confirmation. Measurement of cell ploidy was supported by Dr. Takeshi Yoshizumi.

General Discussion

Discussion

In this thesis, several responses of *Arabidopsis thaliana* to nutrient deficiencies and toxicity were analyzed. Although the original goal of the study was to improve plant tolerance to nutrient stresses, no novel strategy to attach tolerance to nutrient stresses was identified. This study may only be another case to show difficulty to intentionally isolate such novel genes by genetics or to show that only restricted numbers of genes seem to be involved in tolerance to nutrient stresses. Screening of gain-of-function mutants, performed in chapters 5 and 6, was a novel method and I expected to isolate various genes. The failure of the screening to improve tolerance to nutrient deficiency indicated incompleteness of the screening. To complete the screening, I suggested novel strategies. I believe these strategies enable genome-scale screening of gain-of-function mutants.

If picking up the important progresses made in this thesis, an unknown mutation in a low-sulfur mutant was identified in chapter 1, regulation of gene expressions by low-boron and high-boron was clarified at the transcriptome level in chapters 3 and 4, bigger-sized mutants were isolated in chapter 5, and a new phenotype of tetraploid was identified in chapter 6. The high-throughput method established in chapter 2 should be useful to all researchers in genetics because this is one of a few most simplified method of DNA extraction from *Arabidopsis* which requires small amounts of time, labor, and cost.

Several nutrients are deficient in some or many crop fields. Fertilizers are added to these fields to support crop production. Nitrogen and phosphorus fertilization can cause enrichment of these elements in environment and each nutrient can threat species richness (Stevens et al. 2004; Wassen et al. 2005). Another point to stress is that some estimations predict depletion of inexpensive phosphorus ores for fertilizer in the world by 2050 (Vance et al. 2003). Improvement of crop uptake and use efficiency of nitrogen and phosphorus is an important approach to settle these severe problems. There have been several reports showing improvement of these factors through genetic modifications of plants. Maize *Dof1* transcription factor improves nitrogen assimilation when overexpressed in *Arabidopsis* (Yanagisawa et al. 2004). Overexpression of chloroplastic nitrite transporer *CsNitr1-L* also improves nitrogen use efficiency in rice (Sustiprijatno et al. 2006). The only reported genetic modification for improvement of low-phosphorus tolerance is overexpressoin of the *OsPTF1* gene in rice (Yi et al. 2005). Improvement of low-phosphorus tolerance of *Arabidopsis* through overexpression of H^+-transporting pyrophosphatase *AVP1* is also shown (Yang et al. 2007). These modifications will contribute to settle the severe problems above, if they can overcome transgenic problems. At present transgenic crops are not accepted in many countries around the world, and even in the future use of transgenic plants for crop production may be restricted to non-food uses such as

forages and bio-energies.

If completing the list of genetic modifications for improvement of plant tolerance to nutrient deficiencies, LKS1, the regulator of the potassium transporter AKT1 as well as the regulators of LKS1 improves tolerance to potassium deficiency when ectopically overexpressed in *Arabidopsis* (Xu et al. 2006). Apple *sorbitol-6-phosphate dehydrogenase* (*S6PDH*) gene improves tolerance to boron deficiency when introduced into tobacco (Brown et al. 1999). Boron transporter BOR1 also improves tolerance to boron deficiency when overexpressed in *Arabidopsis* (Miwa et al. 2006). Barley *nicotianamine aminotransferase* genes improve tolerance to iron deficiency when overexpressed in rice (Takahashi et al. 2001). Modified yeast ferric reductase improves plant tolerance to iron deficiency (Oki et al. 2004; Ishimaru et al. 2007). Expression of bacterial flavodoxin also rescues plant growth under iron deficiency (Tognetti et al. 2007).

Compared with the large number of various genes involved in plant tolerance to nutrient deficiency (ie, genes whose loss-of-function mutants are sensitive to nutrient deficiency), the number of genes which improve plant tolerance to nutrient deficiency listed above is restricted. Although a few endogenous genes improve tolerance to nutrient deficiencies, this fewness of endogenous genes may indicate that *Arabidopsis* and other plant species evolved nearly fully to adapt to nutrient deficiencies not to allow further tolerance attached by modification of endogenous genes.

A recent study in our laboratory, on the other hand, is showing an interesting phenomenon. The study indicates difference between ectopic overexpression under the control of 35S promoter and activation through combination of 35S enhancer and native promoter of the endogenous boron transporter *NIP5;1* in *Arabidopsis*, and improvement of tolerance to boron deficiency is attached only by activation of this gene under some condition. Overexpressors of *NIP5;1* even tend to be more sensitive to boron deficiency (Yuichi Kato et al., not published yet). If this is true to *NIP5;1*, the same phenomenon could be observed in other nutrient transporters. To test this possibility, activation-tagged lines were obtained from GABI-Kat which have T-DNA insertions of activation-tagging vectors near the promoter regions of sulfate transporter *SULTR1;2* or putative phosphate transporter *PHO1* of *Arabidopsis* here (Yoshimoto et al. 2002; Shibagaki et al. 2002; Hamburger et al. 2002). Growth of probable *SULTR1;2* activator, S2 line, was observed under sulfur deficiency and growth of probable *PHO1* activator, P1 line, was observed under phosphorus deficiency (Fig. D-1). Surprisingly S2 plants appear more tolerant to sulfur deficiency than wild-type and P1 plants appear more tolerant to phosphorus deficiency than wild-type. Although further confirmations are necessary, these results indicate that tolerance to nutrient deficiency is attached to plants only by activating endogenous nutrient transporters. Extensive study of activating all identified nutrient transporters in *Arabidopsis* may identify various endogenous nutrient transporters which improve tolerance to nutrient deficiency.

In activation-tagged lines, gene expressions tend to be activated with its tissue specificity kept as endogenous (Weigel et al. 2000). If expression pattern is kept at single cell levels in

activation lines, nutrient transporters are expressed in its endogenous cells to properly take their roles such as nutrient uptake from media into epidermal cells or xylem loading of the nutrients, which should be preferable for improvement of plant tolerance to nutrient deficiency. From this point of view, screening of gain-of-function mutants should be more effective if we use activation-tagged lines instead of FOX lines. For improvement of screening efficiency, full-length cDNAs should be expressed under the control of the combination promoter consisting of 35S enhancers and native promoters, preferably with sea urchin insulator sequence inserted upstream of these promoters, in novel FOX lines.

After identification of genes which improve plant tolerance to nutrient deficiency, can we modify crops without transgenes? We can find an example in which bread wheat was reverse-genetically improved without transgenes (Slade et al. 2005). In this study, *Waxy* homeologs *Wx-A1* and *Wx-D1* were mutated by TILLING in hexaploid wheat cultivar Express. They succeeded to prepare near-full *waxy* line of segregant possessing each homozygous mutation, because another *Wx-B1* homeolog was originally deleted in Express. Although mutation is randomly inserted in TILLING and most mutations are restricted to C to T or G to A transitions consistent with guanine alkylation (Colbert et al. 2001, Slade et al. 2005), sequential TILLING and backcrossing may enable modification of promoter sequences or modification of codon usage through accumulation of silent mutations in favor of major codons, for activation of gene expressions. Or alternatively, some transposons enhance expressions of nearby genes or may shut down the spreading of heterochromatins along chromosome from original target site of transcriptional gene silencing onto the target genes. For application of basic knowledge in plant genetics to improvement of crop tolerance to nutrient deficiency, there are very challenging but not impossible studies, to be performed to save our future.

References

Brown PH, Bellaloui N, Hu H, Dandekar A (1999) Transgenically enhanced sorbitol synthesis facilitates phloem boron transport and increases tolerance of tobacco to boron deficiency. Plant Physiol 199: 17-20

Colbert T, Till BJ, Tompa R, Reynolds S, Steine MN, Yeung AT, McCallum CM, Comai L, Henikoff S (2001) High-throughput screening for induced point mutations. Plant Physiol 126: 480-484

Hamburger D, Rezzonico E, Petétot JMC, Somerville C, Poirier Y (2002) Identification and characterization of the Arabidopsis *PHO1* gene involved in phosphate loading to the xylem. Plant Cell 14: 889-902

Ishimaru Y, Kim S, Tsukamoto T, Oki H, Kobayashi T, Watanabe S, Matsuhashi S, Takahashi M, Nakanishi H, Mori S, Nishizawa NK (2007) Mutational reconstructed ferric chelate reductase confers enhanced tolerance in rice to iron deficiency in calcareous soil. Proc Natl Acad Sci USA 104: 7373-7378

Oki H, Kim S, Nakanishi H, Takahashi M, Yamaguchi H, Mori S, Nishizawa NK (2004) Directed evolution of yeast ferric reductase to produce plants with tolerance to iron deficiency in alkaline soils. Soil Sci Plant Nutr 50: 1159-1165

Miwa K, Takano J, Fujiwara T (2006) Improvement of seed yields under boron-limiting conditions through overexpression of BOR1, a boron transporter for xylem loading, in *Arabidopsis thaliana*. Plant J 46: 1084-1091

Shibagaki N, Rose A, McDermott JP, Fujiwara T, Hayashi H, Yoneyama T, Davies JP (2002) Selenate-resistant mutants of *Arabidopsis thaliana* identify *Sultr1;2*, a sulfate transporter required for efficient transport of sulfate into roots. Plant J 29: 475-486

Slade AJ, Fuerstenberg SI, Loeffler D, Steine MN, Facciotti D (2005) A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nat Biotech 23: 75-81

Stevens CJ, Dise NB, Mountford JO, Gowing DJ (2004) Impact of nitrogen deposition on the species richness of grasslands. Science 303: 1876-1879

Sustiprijatno, Sugiura M, Ogawa K, Takahashi M (2006) Improvement of nitrate- and nitrite-dependent growth of rice by the introduction of a constitutively expressing chloroplastic nitrite transporter. Plant Biotech 23: 47-54

Takahashi M, Nakanishi H, Kawasaki S, Nishizawa NK, Mori S (2001) Enhanced tolerance of rice to low iron availability in alkaline soils using barley nicotianamine aminotransferase genes. Nat Biotech 19: 466-469

Tognetti VB, Zurbriggen MD, Morandi EN, Fillat MF, Valle EM, Hajirezaei M-R, Carrilo N (2007) Enhanced plant tolerance to iron starvation by functional substitution of chloroplast ferredoxin with a bacterial flavodoxin. Proc Natl Acad Sci USA 104: 11495-11500

Vance CP, Uhde-Stone C, Allan DL (2003) Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytol 157: 423-447

Wassen MJ, Venterink HO, Lapshina ED, Tanneberger F (2005) Endangered plants persist under phosphorus limitation. Nature 437: 547-550

Weigel D, Ahn JH, Blázquez MA, Borovitz JO, Christensen SK, Frankhauser C, Ferrándiz C, Kardailsky I, Malancharovil EJ, Neff MM, Nguyen JT, Sato S, Wang ZY, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation tagging in Arabidopsis. Plant Physiol 122: 1003-1013

Xu J, Li HD, Chen LQ, Wang Y, Liu LL, He L, Wu WH (2006) A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. Cell 125: 1347-1360

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: improved nitrogen assimilation and growth under low-nitrogen conditions. Proc Natl Acad Sci USA 101: 7833-7838

Yang H, Knapp J, Koirala P, Rajagopal D, Peer WA, Silbart LK, Murphy A, Gaxiola RA (2007) Enhanced phosphorus nutrition in monocots and dicots over-expressing a phosphorus-responsive type I H+-pyrophosphatase. Plant Biotech J 5: 735-745

Yi K, Wu Z, Zhou J, Du L, Guo L, Wu Y, Wu P (2005) *OsPTF1*, a novel transcription factor involved in tolerance to phosphate starvation in rice. Plant Physiol 138: 2087-2096

Yoshimoto N, Takahashi H, Smith FW, Yamaya T, Saito K (2002) Two distinct high-affinity sulfate transporters with different inducibilities mediate uptake of sulfate in *Arabidopsis* roots. Plant J 29: 465-473

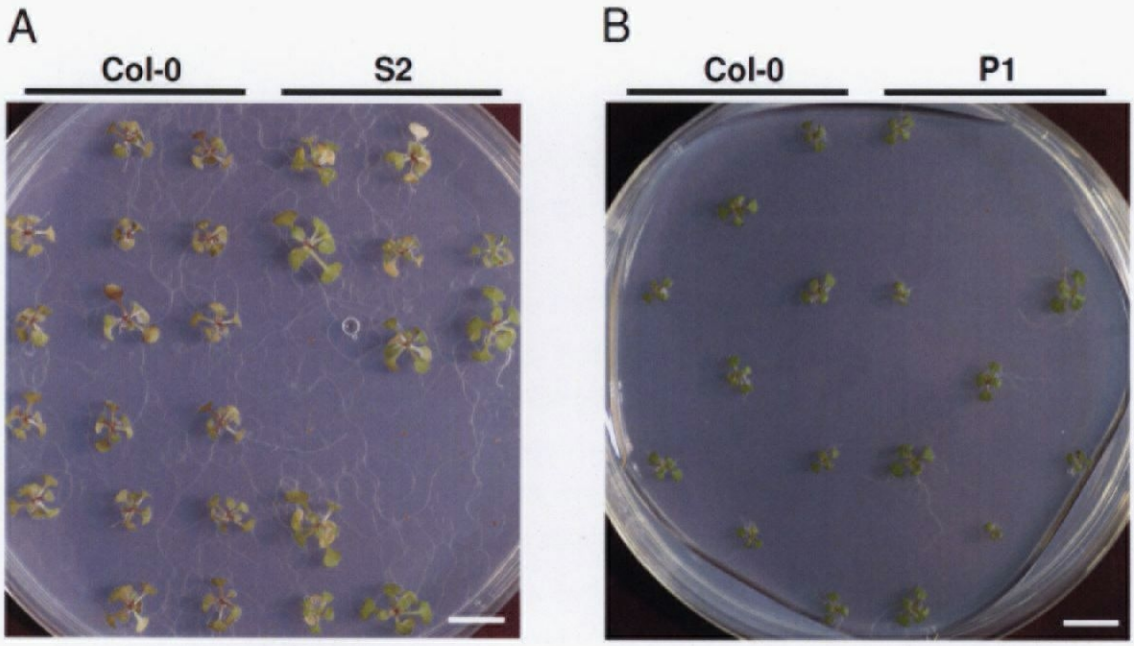

Fig. D-1. Growth of activation-tagged lines. (A) Wild-type (Col-0) and homozygous S2 plants were grown for 18 days on a low-sulfur plate containing 15 μM sulfate. (B) Wild-type and homozygous P1 plants were grown for two weeks on a low-phosphorus plate containing 17.5 μM phosphate. Bars = 1 cm.

Postscript

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