Plant Biotechnology 27, 435–445 (2010)

Original Paper

Possible involvement of ploidy in tolerance to boron deficiency in *Arabidopsis thaliana*

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Received July 2, 2010; accepted July 28, 2010 (Edited by T. Mizoguchi)

Abstract Boron is an essential nutrient for plant growth and reproduction. To identify a novel genetic mechanism which contributes to plant tolerance to nutrient deficiency, we screened *Arabidopsis thaliana* mutants for those tolerant to nutrient deficiencies. One of the isolated lines was tolerant to boron deficiency. This line was designated as LoBT1. From morphological characteristics and ploidy analysis, LoBT1 was found to be tetraploid, although the original screening population was diploid. Because LoBT1 is most likely to be created by the spontaneous duplication of the same diploid genome, LoBT1 is 'autotetraploid'. Independently isolated autotetraploid *A. thaliana* lines which were obtained from the ABRC stock center were also tolerant to boron deficiency, showing that autotetraploidization generally improves tolerance to boron deficiency. Our results represent the first demonstration that autopolyploidization can improve tolerance to environmental stress. Because the clear tolerance was observed under a model system, our observation offers considerable resources to analyze autotetraploid tolerance to environmental stress in more details.

Key words: *Arabidopsis thaliana*, boron deficiency, tetraploid.

Plant genomes sometimes undergo multiplications to generate new plants which have higher ploidy levels than those of their parents. This process is called as polyploidization. Polyploidy is widespread among plant species, with an estimated frequency between 30 and 80% (Masterson 1994). Recent developments in genomics are even revolutionizing the views of angiosperm genomes, and demonstrate that perhaps all angiosperms have likely undergone at least one round of polyploidization (Soltis and Soltis 2009).

The evolutionary significance of polyploidization remains ambiguous. There are two opposing views, one assigning polyploidization a marginal role in evolution and the other granting it a primary creative role. Polyploids may be widespread because they appear repeatedly, without playing a significant role in evolution. Conversely, polyploids may be commonly found because polyploidy promotes adaptive evolutionary changes (Otto and Whitton 2000). Polyploidization is a continuing process even at the moment. From its generality in evolutionary history, it is often speculated as beneficial to plant development, survival and reproduction (Hegarty and Hiscock 2008; Leitch et al. 2005; Masterson 1994; Otto and Whitton 2000; Soltis et al. 2009).

Allopolyploidization is caused by combination of different genomes and is speculated to be beneficial in cases such as niche partitioning and fixation of hybrid vigor. Allopolyploidization between *Arabidopsis* species has been especially a matter of interest and shown to cause rapid physiological changes due to changes in epigenetic regulation and genome rearrangement (Chen 2007; Lee and Chen 2007; Liu and Adams 2007; Osborn et al. 2003; Soltis and Soltis 2000; Wang et al. 2004). On the other hand, except for circumstantial evidence, few clear demonstration has been presented that

Abbreviations: B, boron

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autopolyploidization of plant genome, caused by multiplication of the same genome, bestows any changes in tolerance to stress by direct comparison between diploid and polyploid. This lack of clear evidence has sometimes even lead to a view that autopolyploid plants are not physiologically superior to their unpolyploidized parents.

Epigenetic regulation can also be affected by ploidy levels. Scheid et al. (1996, 2003) detected gene silencing event and epialleles within the same population of colchicine induced autotetraploid *Arabidopsis thaliana*. Transcriptome analysis in allopolyploid plants have uncovered significant expression alterations affecting particular Gene Ontology groups, while only 88 genes were expressed significantly differently between the diploid and the autotetraploid *A. thaliana* (Wang et al. 2006). Some of the genes differentially regulated in allotetraploid are even linked to physiological characteristics (Ni et al. 2009).

In this report we screened gain-of-function mutant and isolated an *A. thaliana* mutant LoBT1 whose root elongation is superior under boron (B) deficiency, although it is not different from that of its parent strain under normal condition. LoBT1 was tetraploid. Not only this spontaneous recovery of low-B tolerant tetraploid, other independently isolated tetraploid lines were also tolerant to B deficiency. Our observation provides the first evidence that autopolyploids are tolerant to environmental stress under a model environment.

Materials and methods

Plant materials

Three LoBT lines were isolated from CS21991 pool of *Arabidopsis thaliana* (L.) Heynh. accession Col-7. Around 10,000 lines of FOX hunting lines (Ichikawa et al. 2006) were also subjected to the screening. Tetraploid lines CS3900 and CS3151 were obtained from Arabidopsis Biological Resource Center and diploid wild-types accession Col-1, Col-7, Landsberg *erecta* (L*er*) were also used.

Plant culture conditions

Surface-sterilized seeds were sown on sterilized MGRL (Molecular Genetics Research Laboratory) growth media (Fujiwara et al. 1992) containing 1% (w/v) sucrose. For preparation of low-B media, borate added to media was reduced and media were solidified with 0.5% (w/v) gellangum (Wako, Osaka, Japan). Agar was not used to prepare gels because of high levels of B contamination. Similarly, plastic bottles were used to prepare media instead of glass bottles. Because distilled water is prepared through glass apparatus, ion exchange water was used. Borate concentration was increased to prepare B-excessive media. For preparation of high-salt media, sodium chloride was added to standard salt and solidified with 1% agar (Wako, Osaka, Japan). 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).

The 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 a 100 μL volume with 8 U each of *Bgl*II, *Xho*I, and *Eco*RI overnight at 37°C using H buffer supplied by the manufacturer (Takara, Kyoto, Japan). The DNA was extracted once with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) vol: vol:vol, once with chloroform, and then precipitated by addition of a 1/10th volume of 3 M sodium acetate and 2 volumes of ethanol. After vortexing, 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. Next, 10 μL of DNA was ligated to the 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 a 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 a 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′). A genomic sequence (5′-…TAC ATT AAT AAA CAC AAG AAC TTT CCA GAT CGA TTG AT-3′) was detected in front of an unknown 8-base sequence (5′-CAA TTC CA-3′) followed by the T-DNA LB sequence (5′-CAG GAT ATA TTC AAT TGT AAA TGG CTT C…-3′).

Co-dominant T-DNA specific primers to detect T-DNA insertions were designed. 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 LoBT1 (Figure 3C). DNA was extracted from leaves as described previously (Kasajima et al. 2004) and subjected to PCR analysis.

Cell ploidy analysis

Cell ploidy was measured as previously described (Yoshizumi et al. 2006). In brief, nuclei were extracted by chopping tissues with a few drops of chopping buffer and stained with CyStain UV precise P (Partec, GmbH, Münster, Germany) following

the manufacturer's protocol. Flow cytometric analysis was performed by a Ploidy Analyzer (Partec, GmbH, Münster, Germany).

Measurement of root-cell length

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

Results

Isolation of an A. thaliana mutant tolerant to B deficiency

To isolate novel genes to improve plant tolerance to nutrient deficiency, *A. thaliana* gain-of-function mutants were screened under nutrient deficiency. Around 400,000 seeds from the pools of activation-tagged lines (Weigel et al. 2000) or FOX hunting lines (Ichikawa et al. 2006) were sown on nitrogen deficient, phosphorus deficient, potassium deficient, B deficient, or B excessive sterilized solid media or grown on urethane sheets with sulfur deficient solution with 0.5-mm separations each other. Tolerant individuals were visually selected and seed gathered (first screening). We will refer the progeny submitted to the first screening to 'S_1 generation' in this paper. S_1 generation corresponds to several generations from the first transformants of activation-tagged lines and the next generation from the first transformant of FOX hunting lines. Several tens of lines were selected in the first screening. Seeds were obtained by selfing these candidate lines (S_2 generation seeds). S_2 seeds were again analyzed for their phenotypes on the same media with the first screening (second screening). Three S_2 lines showed the same phenotypes as their S_1 plants. Phenotypes of all these lines were tolerance to B deficiency. These mutants were designated as LoBT1, LoBT2, and LoBT3 (for Low Boron Tolerance).

Segregation pattern of LoBT2 is complex and segregants showing clear segregation will be picked up for further analysis. LoBT3 was bigger than wild type under normal condition, as well as under B deficiency. Probably because the mutant phenotype was not so vigorous, mapping approach with F_2 plants between accession L*er* is not successful at the moment. Thus, LoBT1 was further analyzed in this study. Figure 1A shows the phenotype and segregation of LoBT1 on low-B medium. Low-B tolerance is segregating in the S_2 progeny of LoBT1. Frequency distribution of root length on low-B medium is shown in Figure 1B. S_2 segregants of LoBT1 were separated into wild types (short root) and mutant types (long root). Of the 60 S_2 plants measured, 28 were classified as long-root and 32 as short-root. This segregation pattern is not consistent with the Mendelian segregation pattern. To further observe the segregation pattern of LoBT1, five short-root S_2 plants and five long-

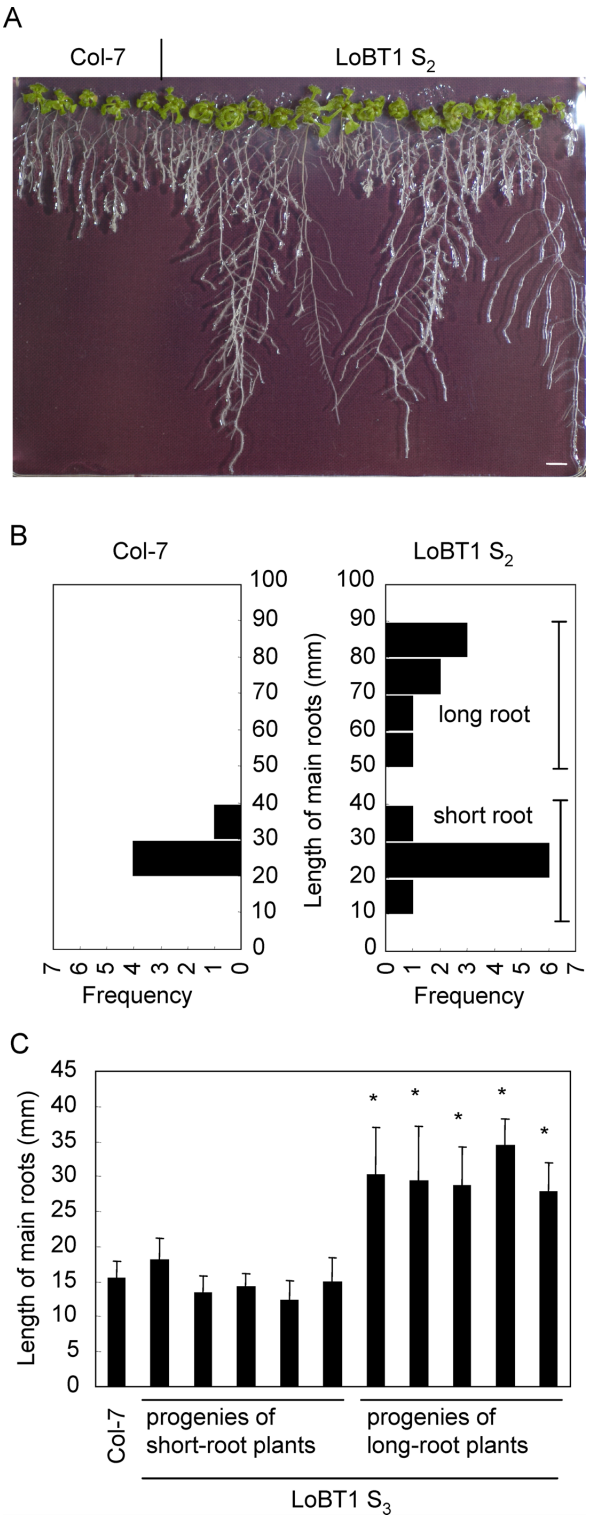

Figure 1. Segregation of LoBT1 in second and third progenies. (A) Wild-type (Col-7) and LoBT1 S_2 plants were grown for 18 days on low-B medium containing 0.2 μM borate. Bar=1 cm. (B) The length of the main roots of plants shown in (A) were measured. Frequency distributions are shown for wild-type and for LoBT1 S_2 plants. (C) Length of the main roots of S_3 progenies. Wild-type (Col-7) and LoBT1 independent S_3 progenies from five short-rooted, second-progeny plants and five long-rooted, S_2 plants were grown for 10 days on low-B media containing 0.2 μM borate and the lengths of the main roots were measured. Means and SDs are shown. n=5~10. Asterisks indicate significant differences from wild-type (Student's *t*-test, $P<0.01$).

root S_2 plants were randomly selected. Self-pollinated S_3 seeds were collected and growth tested on low-B media (Figure 1C). Surprisingly, all S_3 plants showed the same phenotypes as their parents (short or long roots under B deficiency). This segregation pattern is consistent with the hypothesis that the S_1 plant was a chimera of wild-type and low-B tolerant (mutant) tissues.

LoBT1 is tetraploid

Seeds of mutant segregants were larger than those of the wild-types, flowers were also larger and pollen grains also were larger as shown in Figure 2A. Both long and short diameters of pollen grains were greater in LoBT1 mutant-type segregant than in the wild-type ($P<0.0000001$, Figure 2B). These morphological differences are similar to the general morphological characters of the tetraploid plants (Otto and Whitton 2000). To confirm the ploidy of LoBT1, cell ploidy was measured with whole shoots (Figure 2C, D). Compared with the frequency distribution of nuclear DNA amounts in diploid wild-type (Col-7), LoBT1 lacks the left-most peak corresponding to 2C cells, which is clearly detected in the diploid. These characteristics confirm that LoBT1 is tetraploid. Cell ploidy was also checked in several segregating S_2 plants of LoBT1 (Figure 3A). There was a strong relationship between ploidy and root length under B deficiency. Because LoBT1 is an activation-tagged line, a T-DNA was also detected and the insertion site of the T-DNA was confirmed in the last intron of *At3g15410* gene locus. This T-DNA was inserted in all ten segregating S_2 plants tested (Figure 3B), confirming that this T-DNA insertion is not the reason for the enhanced root elongation of LoBT1 under B deficiency. Whether the loss of At3g15410 function accelerated tetraploidization in this mutant is not clear. Gene structures around the T-DNA insertion is illustrated in Figure 3C. Left border (LB) sequence of T-DNA was detected following the genomic sequence in the last intron of *At3g15410*. Judging from the PCR result in Figure 3B, it seems that the opposite side of the T-DNA also consists of LB sequence and a DNA fragment is amplified by primers 015B and 152R. Such case is often observed in our experience.

To confirm the general character of tetraploids, we obtained two additional autotetraploid lines CS3900 (Wang et al. 2004) and CS3151 from the Arabidopsis Biological Resource Center (ABRC: http://www.arabidopsis.org). Ploidies of CS3900 and CS3151 were also reconfirmed by measuring cell ploidy. Similar to LoBT1, root elongations of CS3900 and CS3151 were

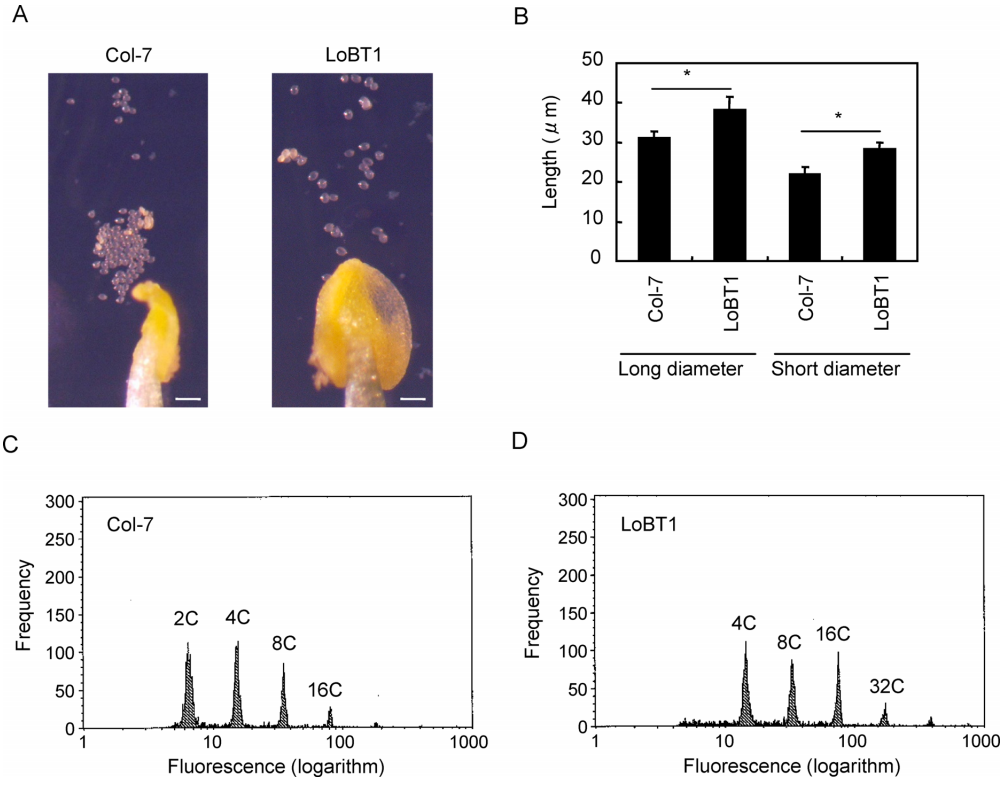

Figure 2. Ploidy of LoBT1. (A) Anthers and pollen of Col-7 and LoBT1 shown to the same scale (bars=100 μm). (B) Pollen size. Long and short diameters were measured. n=20. Means and SDs are shown. Asterisks indicate significant differences by Student's *t*-test ($P<0.0000000001$). (C) Cell-ploidy measurement in wild-types. Whole shoots of normally grown two-week old wild-type (Col-7) plants were measured. Frequency distribution of fluorescence strengths is shown with a logarithmic *x*-axis. Major peaks correspond to 2C, 4C, 8C, and 16C cells. (D) Cell-ploidy measurements in LoBT1 mutant-type segregants. Progenies of a mutant-type segregant were measured. Major peaks correspond to 4C, 8C, 16C, and 32C cells. Approximately 3,000 nuclei were counted in total in both (C) and (D) under the same conditions.

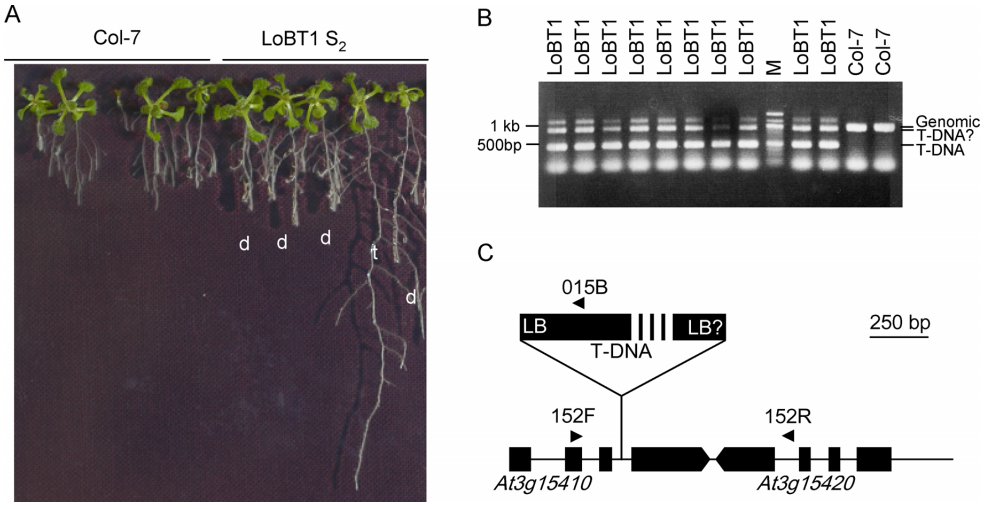

Figure 3. Segregation of ploidy level. (A) Correlation between ploidy and root length under low-B in the segregating S_2 progeny. Wild-type (Col-7) and LoBT1 S_2 plants were grown for two weeks on a low-B plate containing 0.15 μM borate. Cell ploidy was measured in each LoBT1 S_2 plant in the picture with the whole shoot. The results are indicated with the characters 'd' (diploid) or 't' (tetraploid) for each. Bar=1 cm. (B) PCR amplification of T-DNA insertion. M, molecular weight marker. Col-7, wild type. LoBT1, S_2 plants of LoBT1. (C) Gene structure around T-DNA insertion in LoBT1. Exons are shown by black boxes. Only 18th, 19th, 20th and 21st exons are shown for *At3g15410*. *At3g15410* is localized in forward orientation and *At3g15420* is localized in reverse orientation on chromosome 3. T-DNA insertion site and target sequences of the primers are also shown. Direction of the primer sequences are indicated by the direction of the arrow heads.

tolerant to B deficiency (Figure 4A, B). Thus autotetraploid *A. thaliana* is generally more tolerant to B deficiency than diploid *A. thaliana*.

Root elongation under B deficiency

Root elongation pattern was observed more carefully to judge if tetraploid is actually tolerant to B deficiency. Elongation of diploid and tetraploid roots was analyzed under normal and B deficiency conditions (Figure 5). Col-7 and LoBT1 tetraploid S_3 seeds were sown on media containing 30 μM borate (normal conditions, indicated here as 'B30') or media containing 0.15 μM borate (B deficient conditions, indicated here as 'B0.15'). The length of the main roots was measured every day from the start of incubation until the longest root reached the bottom of the vertically orientated plates (Figure 5A). The average length of the tetraploid main roots was slightly longer than that of the diploid ones, although without significant difference. The length of the main roots was significantly greater in the tetraploids under B deficiency. Thus elongation of the main root in the tetraploid is tolerant to B deficiency. The per-plant sum of the lengths of all lateral roots was also measured (Figure 5B). The value was significantly greater in the tetraploid under both normal and low-B conditions. The fractional difference between diploid and tetraploid was nearly twofold under both conditions. Increase in the total lateral root length must be attributable either to an increase in the numbers of lateral roots and/or in their individual lengths. To determine which factor contributes, both values were measured. First, the number of lateral roots equal to or longer than 0.5 mm

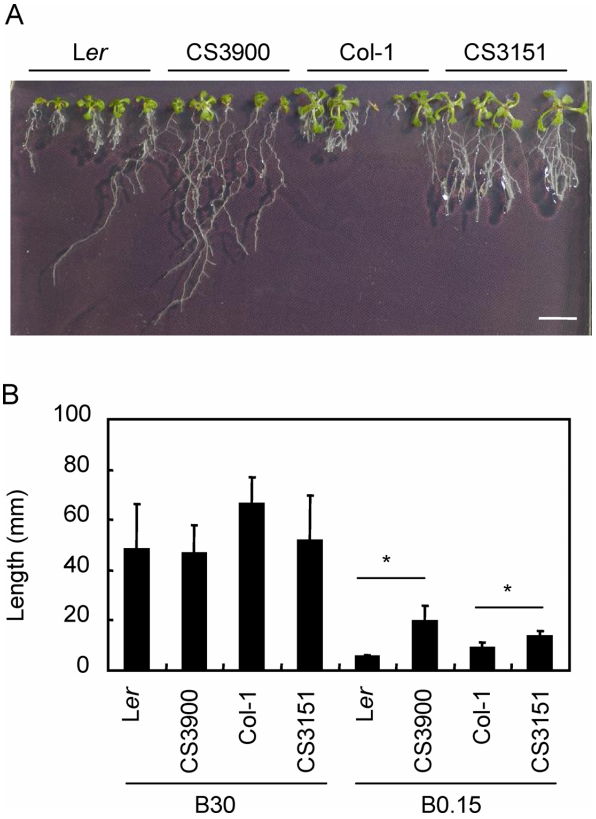

Figure 4. Growth of two additional independent tetraploids. (A) Growth of two other tetraploids under B deficiency. CS3900 and CS3151 are two other tetraploid *A.thaliana* plants obtained from a stock center. The corresponding wild-type diploids are L*er* and Col-1. Plants were grown for two weeks on a low-B medium containing 0.15 μM borate. Bar=1 cm. (B) The same lines were grown on normal (B30) or low-B (B0.15) plates for eight days, and lengths of the main roots were measured. Means and SDs are shown. n=5. Asterisks indicate significant differences between data ($P<0.05$).

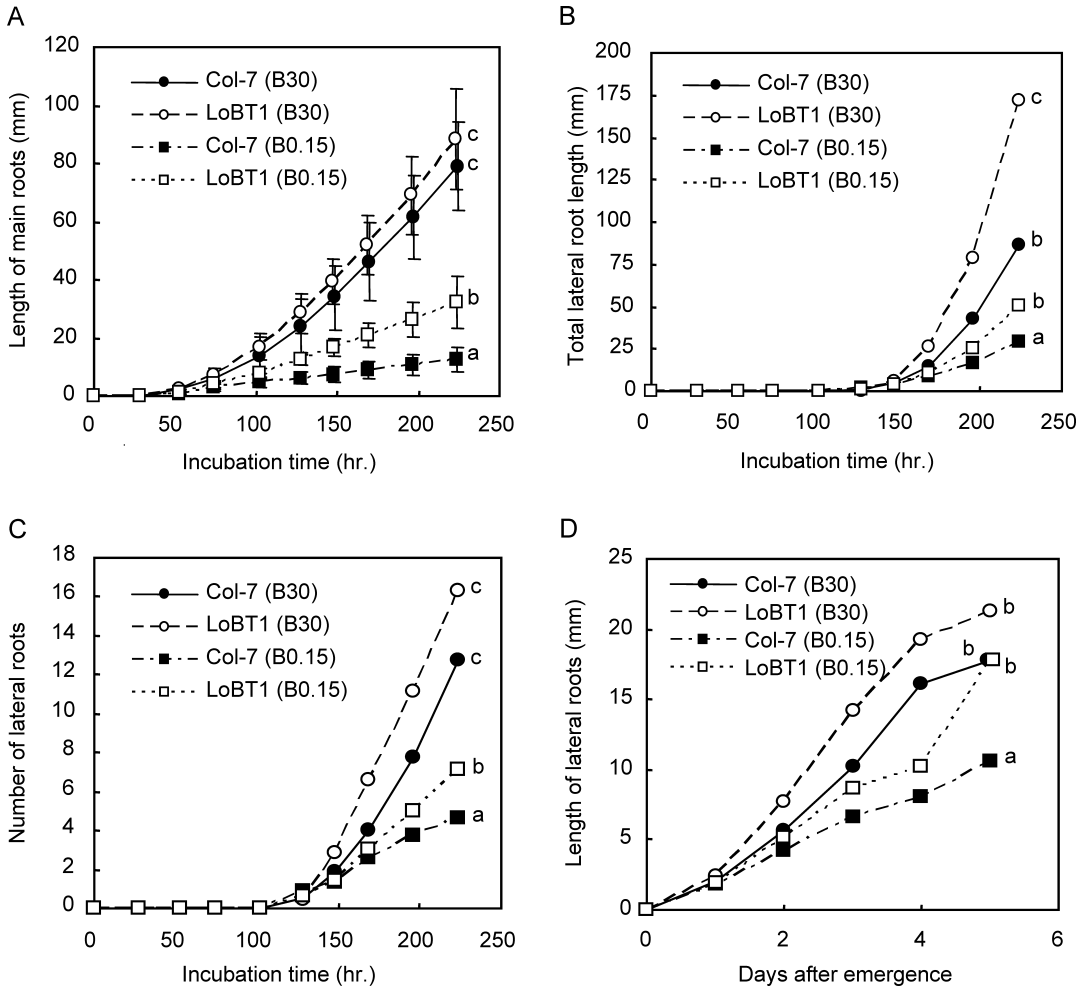

Figure 5. Time-course observation of root elongation. Lengths of main and lateral roots of wild-type diploid (Col-7) and tetraploid (LoBT1) plants were measured for up to nine days, until the longest roots reached the plate margin on the tenth day of incubation. Plants were grown on normal plates containing 30 μM borate (B30) or low-B plates containing 0.15 μM borate (B0.15). The mean values are shown in each graph. Standard deviations are also shown in (A). From (A) to (D), significant differences by Student's *t*-test ($P<0.05$) are indicated by the letters 'a', 'b', and 'c' on the last day in each measurement (a<b<c). (A) Length of the main roots. n=7. (B) The total length of lateral roots per plant. n=7. (C) The number of lateral roots on each plant equal to or longer than 0.5 mm. n=7. (D) Length of lateral roots after emergence. The day when a lateral root reached a length equal to or greater than 0.5 mm was set as the 'first day after emergence'. All available data were used so the replication number (n) varies somewhat between means (n is equal to or greater than 3 for each mean). This is because the dates of 'emergence' differed between the lateral roots.

were counted (Figure 5C). The average number of lateral roots per plant was greater in the tetraploid under both normal and low-B conditions. However, root number increased less than total lateral root length. Second, the lengths of the lateral roots were measured starting from the day when each root emerged and reached 0.5 mm in length (Figure 5D). This measurement could be made for only five days because the lateral roots reached the margins of the plates by that time. The average elongation rate of the lateral roots was also greater in the tetraploids under both normal and low-B conditions. Thus, the great increase observed in the total lengths of the lateral roots in the tetraploids was explained both by increases in the number of lateral roots and also increases in their length. After 5 days from emergence, elongation of the lateral roots in the tetraploids was greater under B deficiency than under normal conditions. Thus, elongation of all roots of the tetraploids is tolerant to B deficiency – the elongation of the main root and also that of the lateral roots.

Cell elongation under B deficiency

Cell length pattern was also observed to characterize low-B tolerance of tetraploid. Suppression of root elongation can be caused by decreased cell proliferation and/or by decreased cell elongation. Under B deficiency, cell elongation is mainly affected, causing corresponding decreases or cessation of root elongation under conditions of this study. To identify the effects of ploidy level on cell elongation, root epidermal cells were measured near the root tip close to the root-hair zone (Figure 6A, B). As evident in the images, decreased cell

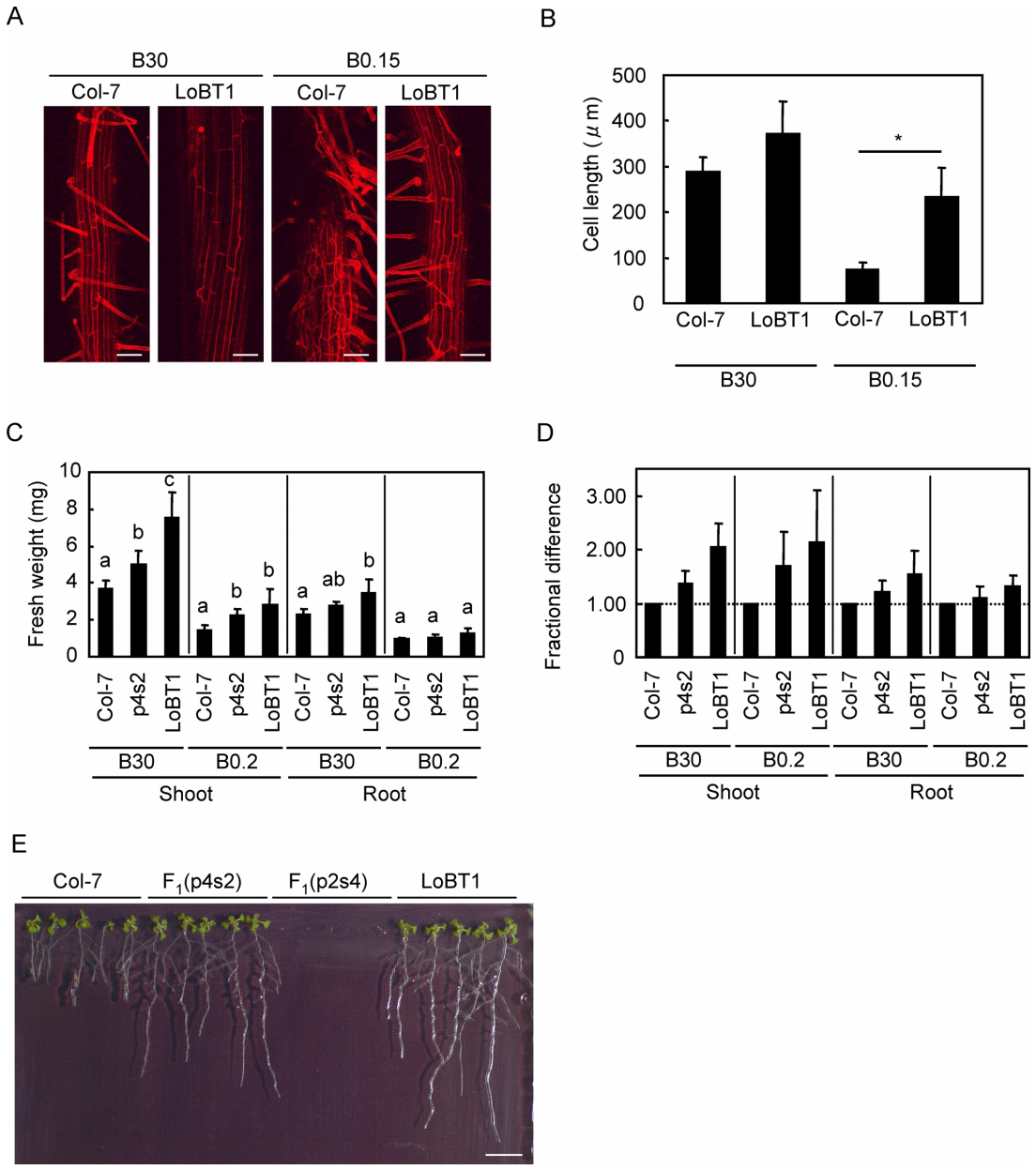

Figure 6. Cell length and biomass. In (A) and (B), wild-type (Col-7) and tetraploid (LoBT1) plants were grown for one week on normal media containing 30 μM borate (B30) or low-B media containing 0.15 μM borate (B0.15). (A) Epidermal cells in the root-hair zone near the root tip were stained with propidium iodide. Bars=100 μm. (B) Longitudal cell-length of epidermal cells measured in the observations above. Means and SDs are shown. n=5. The asterisk indicates significant difference by Student's *t*-test ($P<0.05$). (C) Fresh weights of diploid and polyploids. Diploid (Col-7), triploid (p4s2), and tetraploid (LoBT1) plants were grown for ten days on normal (B30) or low-B (B0.15) media. Fresh weights of whole shoots (Shoot) or whole roots (Root) were measured. The triploid p4s2 line was prepared by crossing LoBT1 pistil with Col-7 stamen. Means and SDs are shown. n=4. Letters indicate significant difference by Student's *t*-test (a<b<c; $P<0.05$). (D) Fractional differences in fresh weights. For data in Figure 6C, fractional differences in fresh weights for each treatment were calculated as the ratio compared with diploid. (E) Plants were grown for ten days on low-B plates containing 0.2 μM borate. Bar=1 cm.

elongation under B deficiency is observed as resulting in shorter cells and more root hairs per unit length of root in the diploids. In the tetraploid, this inhibition of cell elongation by the low-B conditions is reduced, if not completely absent. Thus, tetraploid root cells are more tolerant to the inhibition of cell elongation by B deficiency. Tolerance was also compared with the mean fresh weights (Figure 6C). In this analysis, triploid plants as well as diploid and tetraploid plants were also observed. The triploids were prepared by crossing diploid stamens with tetraploid pistils (p4s2) or vice versa (p2s4), the latter did not germinate even on plates because of parent-of-origin effect in combination with a functional allele of the TTG2 transcription factor (Dilkes et al. 2008). Fresh weights of shoots and roots of the polyploids were greater than those of the diploids under

normal culture conditions. Surprisingly, the ratio of fresh weights of both whole shoot and whole root was not significantly different between the diploids and polyploids under normal conditions or under B deficiency (Figure 6D). This contrasts with the improved root elongation of tetraploid observed under B deficiency (ex. Figure 6E). Thus the growth response of the tetraploid to B deficiency measured by root biomass is not different from that in the diploid. It is deduced that diploid root cells cannot elongate under B deficiency and expand to the horizontal direction from the root axis. Tetraploid cells can elongate to some extent under B deficiency, although it is reduced compared to that under normal condition, along the root axis.

Is tetraploid also tolerant to other stresses?

'Is the tetraploid also tolerant to other stresses?' To answer this question, wild-type diploids and tetraploid segregants of LoBT1 were grown under B toxicity or salt stress, where inhibition of root elongation is evident (Figure 7). Seeds of wild-type diploids and LoBT1 tetraploid S_3 segregants were sown on plates. These were orientated vertically and incubated for two weeks. Almost all plants grown on normal media had roots longer than 10 centimeters. When plants were grown on a B toxic medium containing 5,000 μM B or on a salt toxic medium containing 100 mM NaCl, lengths of the main roots were less than half as long as those grown under the normal conditions. There were no significant differences between the diploids and the tetraploids under either B toxicity or salt stress (Figure 7A). For measurement of shoot fresh weights, plants were grown horizontally on media. Whole shoots of ten plants were weighed for each line (Figure 7B). The fractional difference between the diploids and tetraploids did not greatly change under either B toxicity or salt stress (Figure 7C). Average fractional differences of fresh weights (tetraploid/diploid) were 1.84 under normal conditions, 2.14 under B toxicity and 1.69 under salt stress, without statistical difference. On the other hand, leaf expansion of tetraploid was obviously tolerant to B toxicity on medium containing 6,000 μM boric acid (Figure 7D), although it was not obviously superior under normal condition (Figure 7E). Under this highly B toxic condition, expansion of wild type leaves are inhibited and form callus-like tissue in part. Tolerance of leaf expansion but not of shoot fresh weight of tetraploid under B toxicity may be parallel to the tolerance of root elongation but not of root fresh weight of tetraploid under B deficiency. Although further analysis is necessary to judge tetraploid tolerance to B toxicity, similar response of tetraploid to both B deficiency and B toxicity, if any, is common with the regulation of gene expression (Kasajima and Fujiwara 2007) and growth of *wrky6-3* mutant of *A. thaliana* (Kasajima et al. 2010).

Discussion

Can tetraploidization be beneficial for plant growth if tetraploid is tolerant only to B deficiency? Because B is one of the essential nutrients for plant growth and reproduction, plant growth become worse on B-deficient soils (Cakmak and Römheld 1997). B deficiency has been reported in the field in at least 132 crops and in 80 countries (Shorrocks 1997). Here, the definition of 'B deficient soil' is that plant growth is improved by application of B fertilizer to the soil. Among various physiological disorders caused by B deficiency, sterility and cessation of root elongation occur at relatively moderate B deficient conditions. In areas of such B-deficient soils, root elongation of plants may be limited. Limitation in root elongation may also affect uptake of other nutrients, such as phosphorus and nitrogen, which are deficient in a large part of the soils and are the limiting factors of plant growth. Thus tetraploidization may rescue root elongation in B-deficient areas and secure uptake of various nutrients to improve overall growth.

If the tetraploid is tolerant to B deficiency, we speculated that the diploid might upregulate its cell ploidy to mimic the tolerance of the tetraploid under B deficiency. We measured cell ploidy of whole shoots and whole roots under normal and low-B conditions (Figure 8). In shoots, the incidence of 4C cells decreased by 11% under B deficiency and that of 32C cells (which was about 1.3% of total cells under normal conditions) was not detected under low-B condition. The proportions of cells of the other ploidy levels did not change significantly between normal and low-B conditions in the shoot. On the other hand, a clear change of cell ploidy was observed in the roots where, under low-B conditions, 2C cell incidence decreased by 27%, 4C cell incidence increased by 16% and 8C cell incidence increased by 43%. The incidence of 16C cells seemed to decrease under low-B conditions but this 27% decrease was significant only at $P=0.08$. Considering that tetraploids (which do not have any vegetative 2C cells at all) are tolerant of low-B conditions in terms of their root elongation, this pattern of cell ploidy regulation in diploids under low-B condition could also contribute to the improvement in root elongation under low-B conditions. On the contrary to our study, Reguera et al. (2009) observed clear downregulation of cell ploidy levels by B deficiency in root nodules. Relationship between cell ploidy and B deficiency poses a novel question to be answered.

Entry of nutrients into the cell is expected to be influenced by the ratio of surface area and cell volume. When the cell becomes bigger, its volume increases more rapidly than its surface area and insufficient import leads to growth arrest (Kondorosi et al. 2000). The tolerance

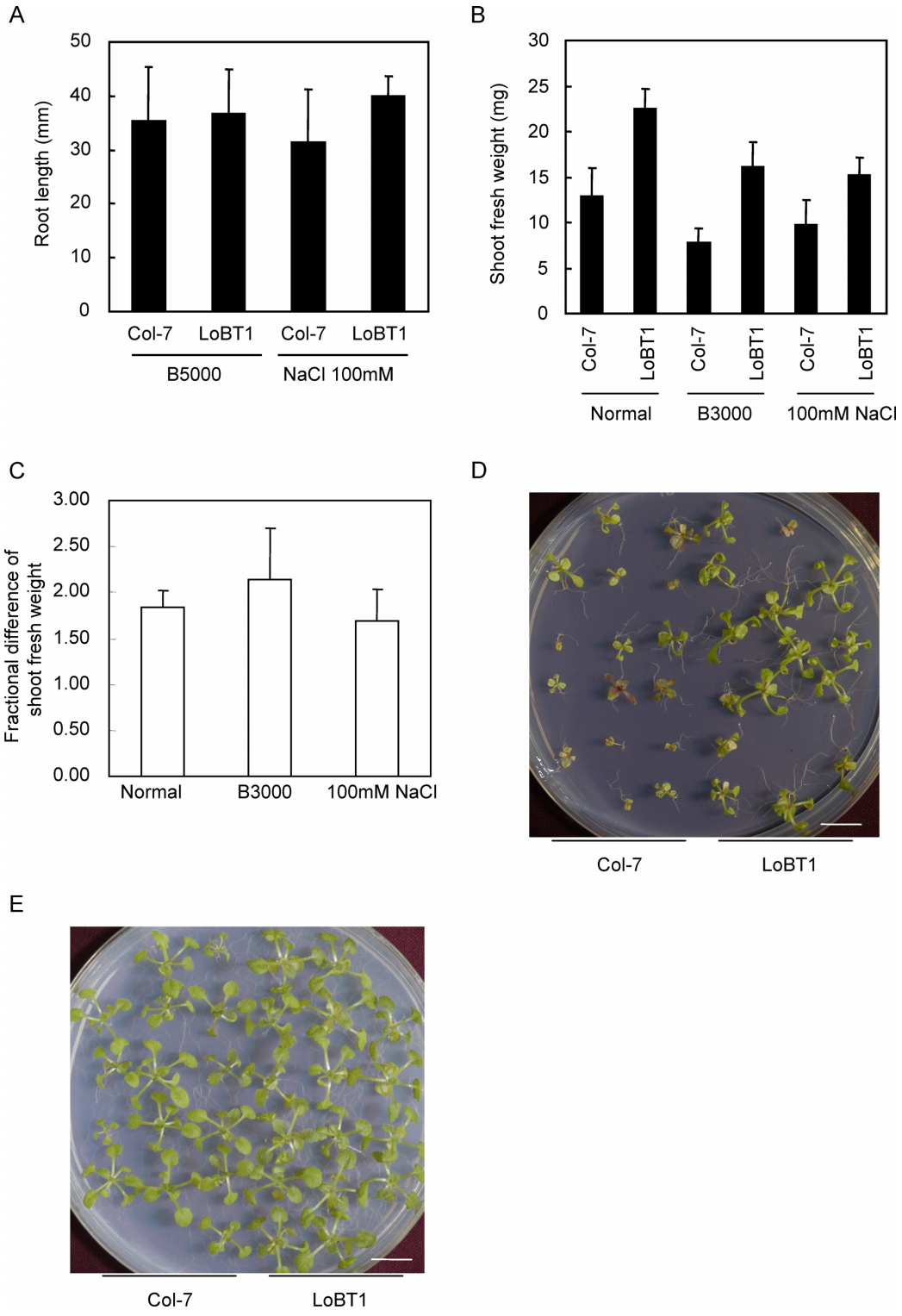

Figure 7. Tolerance to B toxicity and salt stress. (A) Lengths of the main roots. Wild-type diploids (Col-7) and LoBT1 S_3 tetraploid segregants were grown on media containing toxic levels of B (B5000) or salt (NaCl 100 mM). For observation of root elongation, plants were placed vertically and incubated for two weeks. Data represent means and SDs. n=6. Almost all plants grown on normal media had roots longer than 10 centimeters. (B) Fresh weights of the whole shoots. For measurements of shoot fresh weight, plants were incubated horizontally for 18 days on media containing toxic levels of B (B3000) or salt (100 mM NaCl). Data represent means and SDs. n=10. (C) Fold difference of shoot fresh weights. Fold difference (tetraploid/diploid) was calculated for data in (B). n=10. There was no significant difference between data (Student's *t*-test, $P<0.05$). (D) Growth on B toxic medium. Diploids and tetraploids were grown for 2 weeks on medium containing toxic levels of B (B6000). Bar=1 cm. (E) Growth on normal medium. Diploids and tetraploids were grown for 2 weeks on normal medium. Bar=1 cm.

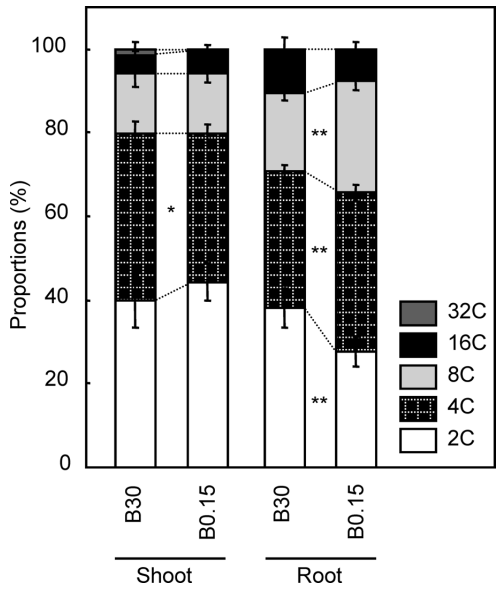

Figure 8. Cell ploidy proportions under normal and low-B conditions. Diploid wild-type Col-7 was grown for two weeks on normal (B30) or low-B (B0.15) plates. The cell ploidy of whole shoots (Shoot) or whole roots (Root) was measured. Means and SDs are shown. In each measurement a total of 3,000 nuclei were counted. Asterisks indicate significant differences by Student's *t*-test ($P<0.05$ for single asterisk, and $P<0.01$ for double asterisks, n=5).

of tetraploid to B deficiency is against this idea. Multiplication of the genome has been proposed to increase metabolic activity, rRNA synthesis and transcriptional activity (Kondorosi et al. 2000). Such benefits from ploidy may result in low-B tolerance in tetraploid. Specific upregulation of B transporter genes is not probable, because no B transporter family genes (NIP family and BOR family) is differentially regulated in the transcriptome comparison between *A. thaliana* diploid and tetraploid (Wang et al. 2006). Recently, we found that WRKY6 affects *A. thaliana* tolerance to B deficiency without changing B content (Kasajima et al. 2010). This observation led us to hypothesize the contribution of some unknown mechanism other than B content to the plant phenotypes under B deficiency. Ploidy may alternatively also rescue this secondary and unidentified stress or signal which is caused by B deficiency.

Tolerance of tetraploid *A. thaliana* was found, closely analyzed and confirmed in this report. Root cell elongation was improved in tetraploid under B deficiency, and this led to improved root elongation of both main root and lateral root in tetraploid under B deficiency. On the other hand, fresh weights were equally affected by B deficiency in both diploid and tetraploid. Thus higher ploidy level rescues root elongation under B deficiency but does not rescue biomass under B deficiency. This differential response of tetraploid suggests differential mechanisms between inhibition of root elongation and reduction of growth rate by B deficiency, and also demonstrates the necessity to observe both root elongation and root biomass to properly estimate root tolerance to environmental stresses such as salinity, heavy metal toxicity, and nutrient deficiencies. Involvement of ploidy level in plant tolerance to B deficiency was a bolt out of the blue. The reason and significance of this phenomenon is a mystery at the moment. Future analysis will identify novel genetic and physiological changes in tetraploid which improve plant tolerance to B deficiency.

Acknowledgements

We acknowledge the ABRC for providing materials used in this study. We thank Tsukasa Shigeta, Yuko Kawara and Mao Sugasawa for technical assistance. We also acknowledge the reviewers for critical comments and advices during the review processes. This work was supported in part by grants from the Japanese Society for the Promotion of Science to I.K., from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomics for Agricultural Innovation Grant IPG-0005 to T.F.), Grants-in-Aid for Scientific Research on Scientific Research (S) and Innovative Areas of The Ministry of Education, Culture, Sports, Science, and Technology, Japan to TF.

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