Chapter 3

Identification of novel *Arabidopsis thaliana* genes which are induced by high levels of boron

Abbreviations

B, boron; MATE, multidrug and toxic compound extrusion; RGI, ratio of gene induction; qPCR, quantitative polymerase chain reaction

Introduction

There are 17 essential elements in plants, including boron (B) (Cakmak and Römheld 1997). Symptoms of B deficiency first appear as growth retardation, particularly in root elongation. B is also necessary for reproductive growth, most notably in flowering, and in fruit and/or seed sets. These processes are in fact more sensitive to B deficiency than vegetative growth (Dell and Huang 1997). B is also toxic to plants when present in high excess, whereby plant growth is inhibited and chlorosis and/or necrosis occur (Nable et al. 1997).

There have been a number of insights into the mechanisms underlying both the requirement for B in plants and the toxicity of this element when present in high quantities. Borate is a constituent of rhamnogaracturonan II complex in the plant cell wall (Matoh et al. 1996). Cross-linking of the rhamnogaracturonan II monomers by borate has also been shown to be essential for leaf expansion (O'Neill et al. 2001). Other roles of B have also been proposed, including the maintenance of the structural integrity of plasma membranes (Cakmak and Römheld 1997). On the other hand, a physiological survey has additionally revealed that B toxicity affects a number of cellular processes and inhibits plant growth (Reid et al. 2004). In a previously reported metabolome study, the accumulated levels of several metabolites were observed to be altered in barley cultivars with differing tolerance levels to high B (Roessner et al. 2006).

To better understand the plant responses to B stress, transcriptome analysis of *Arabidopsis thaliana* was performed in this chapter under both low B and high B conditions. A gene expression profile often provides useful information that furthers our understanding of the responses of various plant mechanisms that facilitate the improvement of plant growth. Examples of this include the induction of *SULTR1;2* expression under conditions of sulfur

deficiency and the induction of a gene encoding *nicotianamine aminotransferase* under iron deficiency (Yoshimoto et al. 2002; Shibagaki et al. 2002; Takahashi et al. 2001). The overexpression of stress-induced genes may also lead to stress-tolerance in some plant species, such as the genes induced by aluminum exposure in *Arabidopsis* or by phosphorus deficiency in rice, both of which result in the improved growth of these plants under these respective conditions (Ezaki et al. 2000; Yi et al. 2005).

Genes induced by B deficiency have been described in tobacco BY-2 cultured cells using a cDNA differential subtraction method (Kobayashi et al. 2004). A microarray analysis with *Arabidopsis* roots has also revealed that the expression of *NIP5;1* is induced by B deficiency. NIP5;1 is a channel that facilitates B flux through the root cell membrane and is required for normal plant growth and development under low B (Takano et al. 2006). To my knowledge, no high B-induced gene was reported.

Results and discussion and methods

Transcriptome analysis was performed using the following experimental conditions and samples. *Arabidopsis* was pre-cultured hydroponically for 38 days under long-day conditions (16-h/ 8-h light/ dark cycle) at 22°C, using standard medium (Fujiwara et al. 1992). The borate levels in the medium were adjusted to 150 µM without affecting the pH and the medium was replaced twice weekly. Following preculture, the plants were transferred to media containing 0.3, 150 or 3000 µM borate, referred to as low-B (–B), control (+B), or high-B (++B) conditions, respectively. The B concentration in the low-B medium is in the range known to cause deficiency symptoms, whereas the B concentration in the high-B medium is at levels that cause toxicity symptoms in wild-type *Arabidopsis* plants. After cultivation for 24 hours in low-B, control or high-B media, whole roots or whole rosette leaves were sampled, immediately frozen in liquid nitrogen, and RNAs were extracted with the RNeasy plant mini kit (Qiagen K.K., Tokyo, Japan). Microarray experiments were conducted in two replicate experiments, each of which analyzed six plant samples (low-B treated roots, control roots, high-B treated roots, low-B treated rosette leaves, control rosette leaves and high-B treated rosette leaves). I thus obtained two sets of microarray data for six different RNA samples.

To examine the induction of a known B responsive gene, *NIP5;1*, mRNA accumulation was monitored via quantitative polymerase chain reaction (qPCR) by Dr. Junpei Takano. These analyses were conducted using a SmartCycler (Cepheid, Sunnyvale, CA, USA) with Ex-Taq R-PCR Version (Takara, Ohtsu, Japan) after reverse transcription with MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and an oligo-d(T)16 primer. The primers used to detect transcripts for *NIP5;1* and *elongation factor 1α* were as described previously (Takano et al., 2006). *NIP5;1* expressions in *Arabidopsis* roots were higher in the RNA samples from low-B treated roots compared with the control roots, confirming that the

low-B treatments in these particular experiments were at appropriate concentrations. Also measured was the B concentration under these culture conditions (Fig. 3-1) using inductively coupled plasma-mass spectroscopy as described previously (Takano et al. 2006). The B levels in the low-B treated roots were approximately half of those in the control roots. In contrast, the B concentration in low-B treated rosette leaves was only 10% lower than the controls. Under high-B conditions, the B concentrations in both roots and rosette leaves were higher than the corresponding controls. However, in contrast to the differences in the B concentrations between roots and rosette leaves under control conditions, there were no significant differences found in these concentrations under high-B conditions.

Transcriptome analysis was performed with a 23k Affymetrix GeneChip at the Microarray Core Facility in the University of Pennsylvania. First genes were selected which gave 'detection *p*-value' of below 0.04 in all RNA samples examined. The detection *p*-value corresponds to the statistical differences between the signal strengths of gene-specific and mismatch oligo-DNAs (Please see 'Statistical algorithms reference guide', Affymetrix: http://www.med.upenn.edu/microarr/Data%20Analysis/Affymetrix/statistical_reference_guide.pdf). As a result of this selection, 12,901 and 12,316 genes were selected in the roots and rosette leaves of, respectively.

Next, the relative gene induction (RGI) was calculated for each gene by dividing the 'signal' value in the low-B or high-B samples with the 'signal' value in the control sample. RGI represents the fold changes of mRNA accumulation under low-B or high-B conditions compared with the control, and were calculated for each set of array experiments.

Then compared were the RGI values obtained from the first and the second array experiments to test for reproducibility. For example, the RGI of *NIP5;1* in low-B roots was 7.2 and 5.8 in the first and second experiments, respectively. In low-B treated rosette leaves the values were 1.0 and 1.0, respectively, suggesting that these experiments were indeed reproducible in terms of the *NIP5;1* induction pattern. For comparison of the overall reproducibility of experiments, fold changes in the RGI values between two replications were calculated for each gene. These fold changes were within a two-fold difference (0.5-2.0) for the vast majority of the genes in low-B treated roots (95%), low-B treated rosette leaves (93%), high-B treated roots (96%) and high-B treated rosette leaves (96%), between the two repeat experiments. Furthermore, 79%, 75%, 82% and 82% of the genes were within 1.5-fold difference in low-B treated roots, low-B treated rosette leaves, high-B treated roots and high-B treated rosette leaves, respectively. RGI values were also calculated for two sets of transcriptome data from the TAIR homepage (http://www.arabidopsis.org), for the purposes of comparison with my current data. In the first data set ('ExpressionSet:1005823533', potassium deficiency treatment), 70% and 44% of RGIs in the potassium-deficient sample were within two- and 1.5-fold differences, respectively. In the second data set ('ExpressionSet:1005823539'; sulfur deficiency treatment), 99% and 91% of RGIs in the sulfur-deficient sample were within two- and 1.5-fold differences, respectively. Although a wide variation was observed, the reproducibility of my present findings is comparable to other transcriptome experiments.

Next compared were the RGIs of low-B and high-B samples for each gene to examine whether any correlation existed between the two. The geometric means of the RGIs from two replicate series of analyses were calculated for all genes under each treatment condition. The log values of these means were then plotted, whereby the x and y values of each spot in the resulting scatter plot represent the RGIs of high-B and low-B samples, respectively (Fig. 3-2A,B). The correlation coefficients between the x and y values were calculated to be 0.37 in the root samples and 0.50 in the rosette leaf samples, suggesting that the genes induced by low and high B are weakly but positively correlated with each other in both the roots and rosette leaves of A. thaliana.

Since a significant fraction of the plant genes examined (4-7%) exhibited different induction ratios showing more than a two-fold difference between my replicate experiments, I confirmed the upregulation of mRNA accumulation for a number of key B-responsive genes that had been identified by microarray. I selected genes that satisfied both the following two conditions (formulae 1 and 2):

$$\{\min(R_{L1}, R_{L2}) > 2.0 \cap \mathrm{avg}(R_{L1}, R_{L2}) > 2.5\} \cup \{\min(R_{H1}, R_{H2}) > 2.0 \cap \mathrm{avg}(R_{H1}, R_{H2}) > 2.5\} \quad \cdots\cdots (1)$$

$$\max\{\mathrm{avg}(R_{L1}, R_{L2}), \mathrm{avg}(R_{H1}, R_{H2})\} > |\min\{\mathrm{avg}(R_{L1}, R_{L2}), \mathrm{avg}(R_{H1}, R_{H2})\}|^3 \quad \cdots\cdots\cdots\cdots (2)$$

R_{L1}: RGI in low-B sample in the first experiment
R_{L2}: RGI in low-B sample in the second experiment
R_{H1}: RGI in high-B sample in the first experiment
R_{H2}: RGI in high-B sample in the second experiment
avg: geometric mean
min: minimum value
max: maximum value

With formula (1), genes were picked up whose mRNA accumulations are induced by low-B or high-B, or both. With formula (2), genes were eliminated whose mRNA accumulations are induced by both low-B and high-B. These equations allowed to identify genes specifically induced by low-B or high-B by selecting data from the gray areas shown in Fig. 3-2C.

I also identified five additional genes whose upregulation may be specifically induced in high-B treated rosette leaves. The expression levels of these genes were detectable in high-B treated rosette leaves, but not in the control or low-B treated rosette leaves, and thus did not satisfy the above mentioned criteria. In total, 12 genes were selected and the expression levels of each were examined by qPCR. Ten of these genes gave reproducible results (data not shown). For the determination of mRNA accumulation for these 10 genes, I used the following primers. *At5g57340* (5'-CCATCAGACATACAATGCAAGC-3' / 5'-TCGAGGTCTATGCCTGAACA-3'),

At1g03770 (5'-TCTCGGGAGCTTAGAGGGTA-3' / 5'-ATCTTGCAGGCTTTGCATCT-3'), *At2g04050* (5'-CGTTTCCGGGTTCAGTATTT-3' / 5'-CAGGGTCTTGACCGAGAGAG-3'), *At5g51440* (5'-TCAAACCGACATGTTTCTCG-3' / 5'-TCACGTTCCAACCACGTCTA-3'), *At2g04070* (5'-TGTCTCCGGTTTCAGCATTA-3' / 5'-TGTTAGAGGAAATTGCGGAGT-3'), *At2g41730* (5'-GTCACCAAGGCATCGTAAGG-3' / 5'-TCCGGTGGTATTTGAATGGT-3'), *At2g21640* (5'-CAGGAAGAGGGTGAAGGATG-3' / 5'-CTTGGAGAAGCTCCCGAATA-3'), *At2g04040* (5'-CGCTCCTATGGCCACTGT-3' / 5'-CAAGTGCACCCACTAATCCA-3') and *At1g32870* (5'-AAGAAAGATCCGTCGGAAAAA-3' / 5'-CCAATAGCCACGTTCAGTAGC-3'), in addition to the previously described primers for *NIP5;1* and *elongation factor 1α* (Takano et al., 2005). The fold changes in the expression levels of these genes under low-B or high-B are shown in Table 3-1, including their annotations. Gene annotations are based on the information from either ANNOME (http://www.kazusa.or.jp/katana/annome.html) or NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast).

Among the 10 genes selected, the only gene that was found to be specifically induced in low-B treated roots was *NIP5;1* (Table 3-1). *At5g57340* and *At1g03770* are specifically induced in high-B treated roots, of which *At1g03770* is predicted to encode a zinc finger transcription factor (Table 3-1). Genes *At2g04050*, *At5g51440*, *At2g04070*, *At2g41730*, *At2g21640*, *At2g04040* and *At1g32870* were specifically induced in high-B treated rosette leaves (Table 3-1). These genes include three multidrug and toxic compound extrusion (MATE) family transporter genes, a heat shock protein, and a transcription factor.

I next analyzed the time course of mRNA accumulation for these genes under low-B or high-B conditions, compared with their expression profiles under control conditions. Wild-type *Arabidopsis* plants grown in control media for 38 days were transferred to either low-B or high-B media. After incubation for 6, 24, 48 and 96 hours, RNA was extracted from either whole roots or whole rosette leaves and analyzed by qPCR. In low-B treated roots (Fig. 3-3A), *NIP5;1* transcripts were observed to be the most highly elevated after 6 hours of transfer to low-B medium, and these levels were maintained over a four day period. It is noteworthy that this induction ratio differs from the findings of a previous report (Takano et al., 2006). Hence, although this analysis was performed only once in my present study, it is possible that subtle differences in the experimental conditions, such as plant density and timing of the sampling, have caused this discrepancy. The expression of *NIP5;1* did not alter over four days after high-B treatment (Fig. 3-3B).

The accumulation of *At5g57340* and *At1g03770* transcripts in *Arabidopsis* roots occurred after six hours of treatment in high-B medium (Fig. 3-3B), and was maintained at these two- to three-fold higher levels over four days. In contrast, the upregulation of *At5g57340* and *At1g03770* mRNA in low-B treated roots did not seem to be induced over four days (Fig. 3-3A). These are the first reported examples of high B-induced genes in roots. *At1g03770* is predicted to encode a zinc finger family transcription factor and it is thus possible that this gene regulates the expression of downstream genes that are responsive to high-B.

The accumulation of *At2g04050*, *At5g51440*, *At2g04070*, *At2g41730*, *At2g21640*,

At2g04040 and *At1g32870* gene transcripts in high-B treated rosette leaves was most pronounced after 2 or 4 days of incubation in high-B medium (Fig. 3-3D,E). In low-B treated rosette leaves, inductions of up to 2.2-fold and downregulation by as much as 50% could be observed for these genes (Fig. 3-3C). However, these changes seem to be minor effects in comparison with the expression level changes in the high-B treated rosette leaves. These are the first examples of high B-induced genes in rosette leaves, among which *At2g04040*, *At2g04050* and *At2g04070* are predicted to encode MATE transporters and are located in close proximity to each other on chromosome 2. It is thus possible that chromosome remodeling is involved in regulation of gene expression under high-B. However, it should also be pointed out that in my microarray analysis, the signals from *At2g04060* and *At2g04063*, which are the genes located between *At2g04050* and *At2g04070*, could not be detected even in high-B rosette leaves (data not shown).

In summary, a number of novel high B-induced genes have been identified which include a heat shock protein and a number of MATE family transporters. Further studies will elucidate the roles of these genes in response to B toxicity. It is also possible that the characterization of these genes will lead to the identification of novel physiological response pathways to B toxicity in plants.

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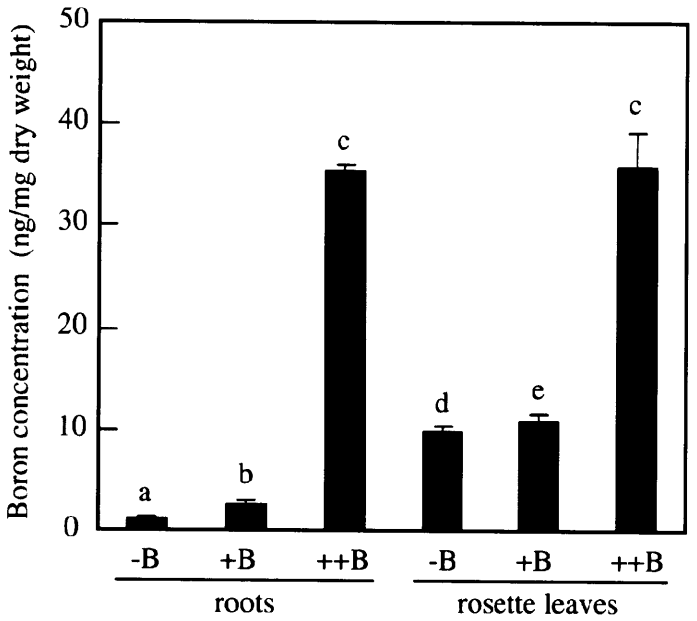

Fig. 3-1. The accumulated levels of B in roots and rosette leaves under various B concentrations in the growth media. *Arabidopsis* plants were grown under low-B (-B), control (+B) and high-B (++B) conditions as described in the text. Whole roots and whole rosette leaves were sampled and heat-dried, and then digested with nitrate. The B concentrations were determined using inductively coupled plasma–mass spectroscopy (n = 3). The letters above each bar indicate groups showing significant differences ($P < 0.05$) between each other ($a < b < d < e < c$).

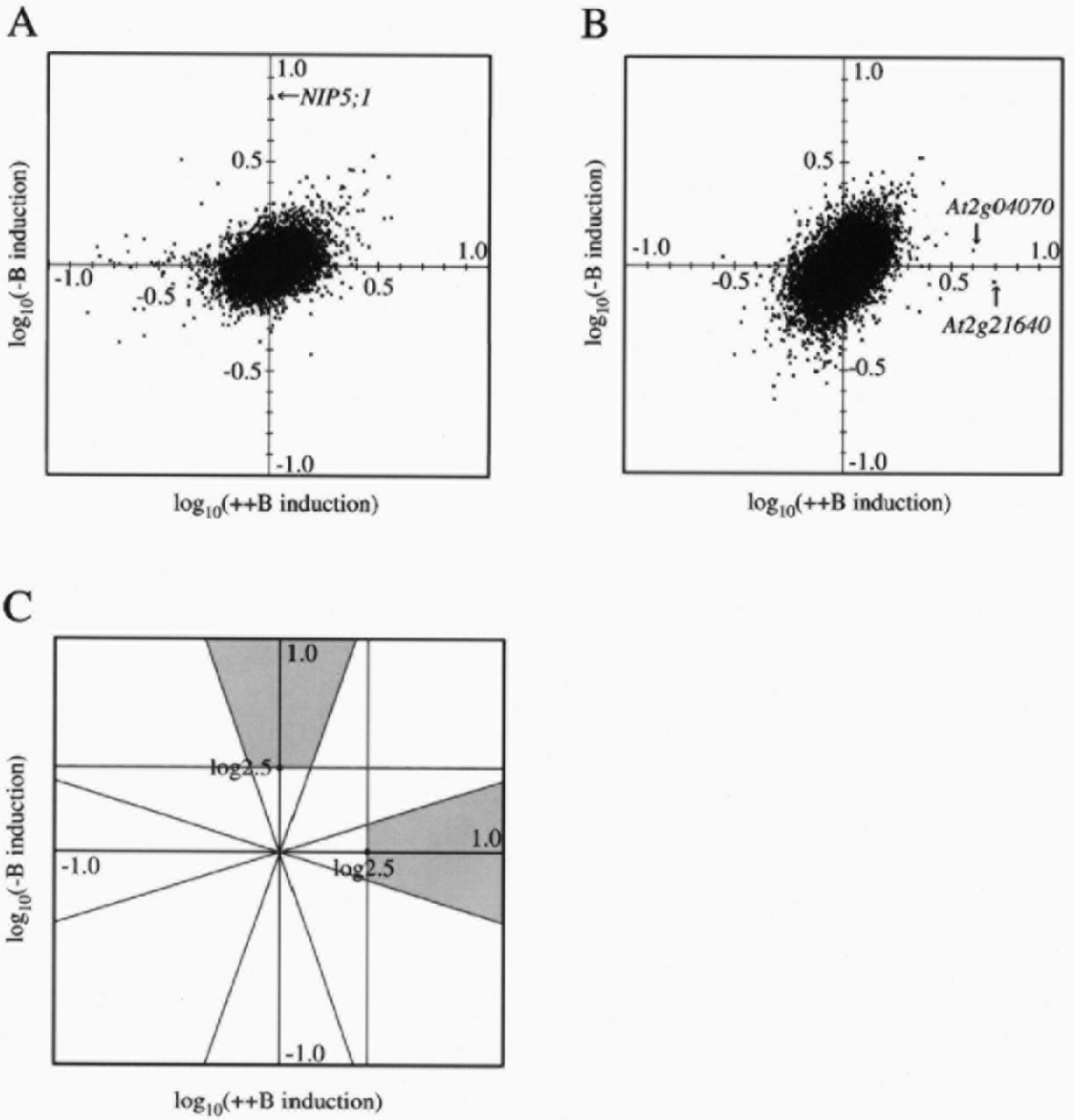

Fig. 3-2. Correlation between the ratios of gene induction following B deficiency and B toxicity. Each spot denotes a single gene, and scatter plots were generated using the $\log_{10}$ values of the geometric RGI means of two replicate experiments for high-B (x values) and low-B samples (y values) from the roots (A) and rosette leaves (B) of *Arabidopsis*. The spots representing *NIP5;1*, *At2g04070* and *At2g21640* are indicated by arrows. (C) Gene selection was performed using the indicated gray areas in which the data satisfy the selection formulae (1) and (2). The formulae for the lines in this figure are x=log2.5, y=log2.5, y=-3x, y=-1/3 x, y=1/3 x, and y=3x.

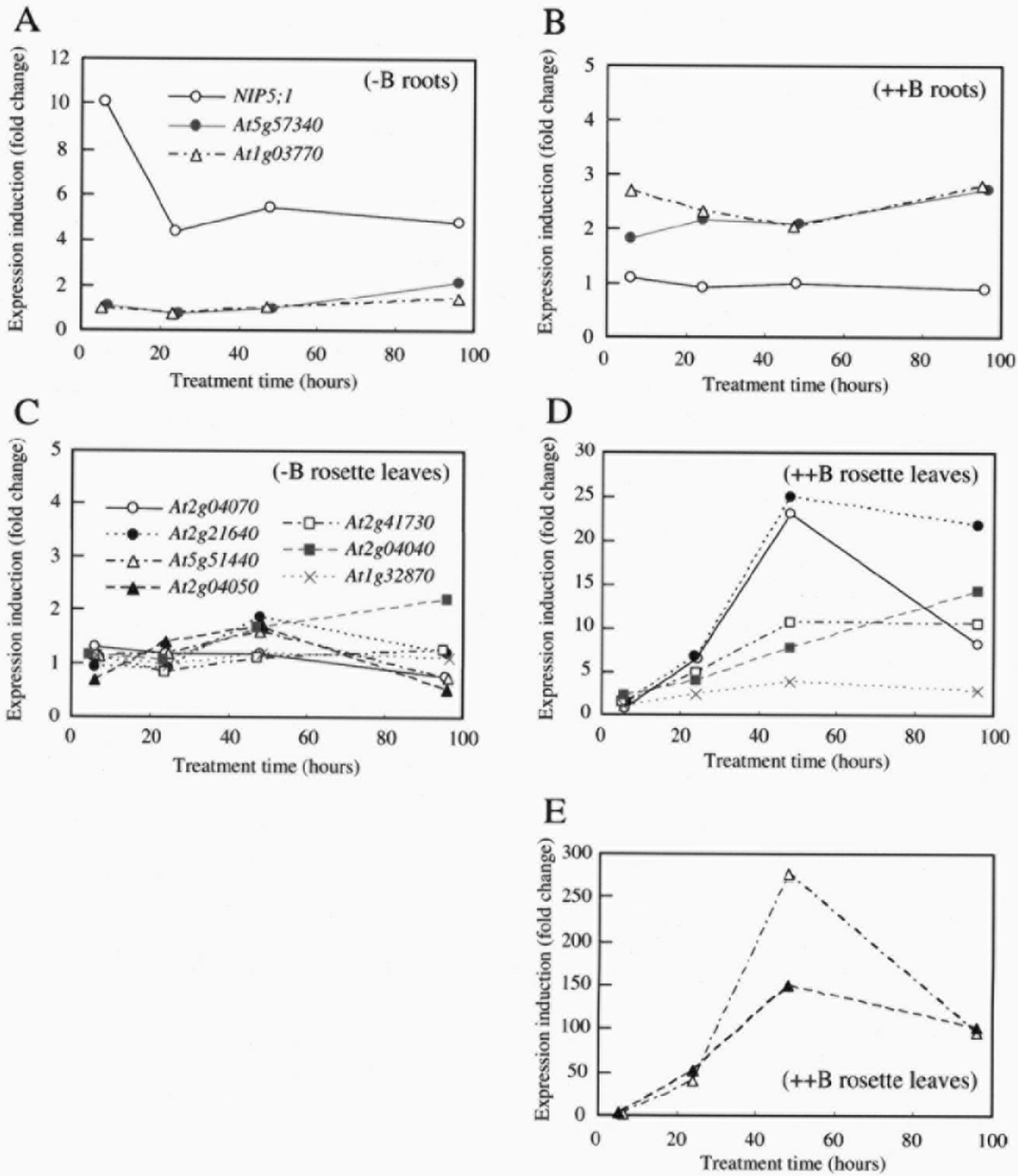

Fig. 3-3. Time-course analysis of mRNA levels of *Arabidopsis* genes after exposure to low-B and high-B. *Arabidopsis* was hydroponically cultured for 38 days in normal media followed by transfer to either low-B, control or high-B media. Plants were then cultured for a further 6, 24, 48 or 96 hours prior to sampling. mRNA levels were determined by qPCR and normalized to *Elongation Factor 1α*. The relative changes in the mRNA levels were based upon the control conditions. (A) low-B treated roots. (B) high-B treated roots. (C) low-B treated rosette leaves. (D) and (E) high-B treated rosette leaves (n = 1). The genes analyzed in (A) and (B) are indicated in (A) and those shown in (C-E) are indicated in (C).

Table 3-1. Induction of selected *Arabidopsis* genes by B nutrition. *P < 0.05, versus controls. n = 3.

AGI code	++B induction	-B induction	Annotation
(roots)			
At5g57340	3.1 ± 0.4*	0.9 ± 0.2	unknown
At1g03770	2.2 ± 0.3*	0.9 ± 0.1	zinc finger family
At4g10380	1.1 ± 0.2	5.4 ± 0.9*	*NIP5;1*
(rosette leaves)			
At2g04050	18.5 ± 8.4*	1.0 ± 0.1	MATE family
At5g51440	14.5 ± 6.0*	1.6 ± 0.5	heat shock protein –like
At2g04070	5.8 ± 1.2*	1.3 ± 0.5	MATE family (*DTX1*)
At2g41730	5.5 ± 1.2*	1.0 ± 0.1	unknown
At2g21640	4.1 ± 0.9*	1.1 ± 0.1	unknown
At2g04040	2.9 ± 1.1*	1.1 ± 0.8	MATE family
At1g32870	2.0 ± 0.1*	1.2 ± 0.0	NAC domain -containing

Acknowledgements

Expression analysis of *NIP5;1* in array samples was performed by Dr. Junpei Takano. Microarray analysis was performed by the Microarray Core Facility in the University of Pennsylvania.

Chapter 4

Regulation of gene expression by boron deficiency around root tip of *Arabidopsis thaliana* and involvement of *WRKY6* in regulation

Abbreviations

ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC oxidase; ACS, ACC synthase; B, boron; PCR, polymerase chain reaction; *GUS*, *β-glucuronidase*

Introduction

Boron (B) is one of the essential elements for plant growth and reproduction (Cakmak and Römheld 1997). Plants have adaptation mechanisms to various nutrient deficiencies including B deficiency. A few responses to B deficiency at molecular levels are reported. *Arabidopsis thaliana* BOR1 protein, a B transporter, accumulates only under B deficiency, which facilitates B translocation from root to shoot (Takano et al. 2005). The first gene whose expression was known to be induced by low-B in *Arabidopsis* was *NIP5;1*, another B transporter (Takano et al. 2006). Several low-B induced genes are also reported in tobacco BY-2 cultured cells using a cDNA differential subtraction method (Kobayashi et al. 2004). In chapter 3, transcriptome analysis was performed under both low-B and high-B conditions. In this analysis no other gene than *NIP5;1* was identified whose expression is highly (more than 2.5-fold) and specifically induced by low-B (not by high-B) after one-day treatment, although expressions of some genes were induced in low-B treated tissues to some extent (Kasajima and Fujiwara 2007). In this chapter, lines that have T-DNA insertions in the upregulated genes by low-B or high-B identified in chapter 3 were selected. Growth of these lines was observed under low-B or high-B conditions, to know the functions of these genes. After observation, *WRKY6* which encodes a transcription factor was picked up and analyzed. Transcriptome analysis in this chapter also identified genes which are induced by long-term B deficiency around the root tip.

Results

Isolation of *wrky6-3* mutant

To identify functions of low-B or high-B induced genes (Kasajima and Fujiwara 2007), several genes were selected whose transcript accumulations are elevated under low-B or high-B, or both. T-DNA insertion mutants were obtained for these genes and growth of homozygous segregants were compared with that of wild-type on low-B and high-B media. Among several mutants, I focused on SALK_12997 (designated as *wrky6-3* in this study), because only this line showed differential growth from wild-type.

In *wrky6-3* mutant a T-DNA is inserted in the third exon and the third intron of *WRKY6* (*At1g62300*), which encodes a transcription factor associated with regulation of gene expressions in response to senescence and pathogen attack (Robatzek and Somssich 2001, 2002). T-DNA in *wrky6-3* causes addition of aberrant five amino acids and truncation following the original 185th tyrosine (Fig. 4-1A). mRNA accumulations were quantified in wild-type and *wrky6-3* seedlings (Fig. 4-1B). I prepared two sets of primers, W1 and W2, to amplify *WRKY6* cDNA. W1 primer set amplifies 5' transcript (between 1st and 2nd exons) and W2 primer set amplifies 3' transcript (between 5th and 6th exons). Accumulatoin of both 5' and 3' transcripts were induced by B deficiency in wild-type. 5' transcript was also detected in *wrky6-3* as in wild-type, although accumulation of 3' transcript was much lower in *wrky6-3*. Expression of *NIP5;1* is induced in wild-type under B deficiency as previously described (Takano et al. 2006). *NIP5;1* transcript accumulation is also induced in *wrky6-3*, indicating that *WRKY6* is not involved in the regulation of *NIP5;1* expression.

Growth of *wrky6-3* is shown in figure 4-2. Growth varied among several independent experiments. Growth was often not different between wild-type and *wrky6-3*, although sometimes there was clear difference in growth under B deficiency. This occasionally different phenotype was also observed in *wrky6-1* and *wrky6-2* mutants in a few cases of several observations (please see materials and methods for line properties). Growths of differently growing wild-type and *wrky6-3* are shown in figure 4-2A. In this experiment roots of *wrky6-3* were shorter than wild-type only on the low-B plates (Fig. 4-2B). To identify the reason for decreased root elongation in *wrky6-3* under B deficiency, lengths of epidermal cells of main roots were measured in several regions, for example a region below hypocotyle (top region) and root-hair zone near the root tip (tip region) (Fig. 4-2C). Several regions of the roots were observed. There was no statistical difference between wild-type and *wrky6-3* on normal media, although wild-type tends to have longer cells than *wrky6-3*. There was also no statistical difference on low-B media, indicating cell proliferation and not cell growth is sometimes inhibited in *wrky6-3* under B deficiency.

Locus of *WRKY6* expression

In previous reports, roles of *WRKY6* in the responses to senescence and pathogen attack in

leaves were analyzed (Robatzek and Somssich 2001, 2002). *WRKY6* promoter activity is induced by these treatments in leaves. Although the promoter activity of *WRKY6* is constantly observed in root, especially in seedlings, no role of *WRKY6* in root has been described. Transgenic plants expressing *β-glucuronidase* (*GUS*) as a reporter under the control of *WRKY6* promoter were grown under B deficiency, and *GUS* expression was observed. GUS staining was not observed in the shoot and observed some staining in root of normally cultivated seedlings as described previously (Fig. 4-3A; Robatzek and Somssich 2001, 2002). Under B deficiency, stronger GUS staining was observed near the root tip (Fig. 4-3B). GUS staining was observed in all outer cell layers of low-B root tip, such as epidermis, cortex and endodermis, including root hairs (Fig. 4-3C).

Transcriptome analysis around root tip

Expression of *NIP5;1*, a B transporter which facilitate B uptake and improve total growth including root elongation under B deficiency (Takano et al. 2006), was not largely different in a *WRKY6* knockout mutant (Fig. 4-1B). B accumulations in both whole roots and whole shoots were also measured under normal and low-B conditions, and there was no statistical difference in B contents between wild-type and *wrky6-3* (data not shown).

To know the effect of *WRKY6* on response to B deficiency, transcriptome analyses were carried out in root tip, where promoter activity of *WRKY6* is activated by B deficiency. Wild-type (C) and *wrky6-3* (W) were vertically cultivated for 9 days on media containing 30 μM (normal, +) or 0.1 μM (low B, -) borate. Root tips of about 8-mm tip portions were sampled and subjected transcriptome analysis with Affymetrix 23k GeneChip by the laboratory of Dr. Masami Yokota Hirai (RIKEN PSC). Experiments were performed in two replications. For data analysis, genes which gave detection *p*-values of less than 0.06 in all experiments were selected. The detection *p*-value corresponds to the statistical differences between the signal strengths of gene-specific and mismatch oligo-DNAs (Please see 'Statistical algorithms reference guide', Affymetrix: http://www.med.upenn.edu/microarr/Data%20Analysis/Affymetrix/statistical_reference_guide.pdf). As a result of this selection, 14,774 genes were selected for further analysis. For comparison of the overall reproducibility of experiments, fold changes in the induction ratios of signal values by low-B between two replications were calculated for each gene. The fold changes were within a two-fold difference (0.5-2.0) for the vast majority of the genes in wild-type (99.1%) and *wrky6-3* (98.6%) between two replications. Furthermore, 94.0% and 92.0% of the genes were within 1.5-fold difference in wild-type and *wrky6-3* respectively. These values are comparable with other transcriptome experiments (chapter 3, Kasajima and Fujiwara 2007).

Patterns of gene expressions

Using averages of signal values of each set of two replications, gene expressions were monitored (Fig. 4-4). Effect of *WRKY6* on gene expression was monitored by comparing the division of W+ signal (normally cultivated *wrky6-3*) by C+ signal (normally cultivated wild-type)

(Fig. 4-4A). Forty five genes were upregulated by more than 2.0-fold by the *wrky6-3* mutation and 7 genes were downregulated to less than 0.5-fold. The most upregulated gene by *wrky6-3* mutation was *At4g19690*, whose expression was 6.0-fold in *wrky6-3* compared with that in wild-type. The most downregulated gene was *At1g80530*, whose expression was 0.11-fold in *wrky6-3*.

Regulation of gene expression by B deficiency in wild-type was monitored next (Fig. 4-4B). 1,193 genes were upregulated by more than 2.0-fold and 333 genes were downregulated by less than 0.5-fold by low-B in wild-type. The top-10 list of up- or down-regulated genes are shoen in table 4-1. Comparison of regulations by low-B in wild-type was also made between the previous study (Kasajima and Fujiwara 2007) and the present study (Fig. 4-4C). In the previous study, wild-type plants were hydroponically cultivated for 38 days with culture containing 150 μM borate, then transferred to cultures containing 150 μM (+) or 0.3 μM (-) borate for 24 hours, and whole root was sampled. Major differences in the present study compared with the previous study were prolonged treatment with low-B, restricted locus of plant tissue (root tip vs. whole root), and lower B content, all of which cause more severe B deficiency. In this comparison, it is clear that much more genes are upregulated by B deficiency under the condition of the present study. Finally, comparison was made of gene expressions under B deficiency between wild-type and *wrky6-3* (Fig. 4-4D). Regulation of gene expression is shown as *x* value and division of *wrky6-3* signal by wild-type signal under low-B in shown as *y* value in the figure. Genes tend to be plotted near the *x* axis, indicating no involvement of *WRKY6* on overall gene expression under B deficiency. Although some part of genes are plotted to positions remote from *x* axis, and so expressions of these genes are up- or down-regulated by *wrky6-3* mutation under B deficiency. The same color labels applied in figure 4-4A (orange label for genes upregulated more than 2.0-fold by *wrky6-3* mutation under normal condition and green label for genes downregulated less than 0.5-fold by *wrky6-3* mutation under normal condition) were also applied to the plots of the same genes, to discriminate between genes which are constantly up-/down-regulated by *wrky6-3* mutation and genes which are up-/down-regulated by *wrky6-3* only under B deficiency. For example, expression of genes which are plotted in the fourth quadrant and remote from both *x* and *y* axes, and not labeled by colors (so the spots are black) are upregulated by B deficiency in wild-type and this upregulation is inhibited by *wrky6-3* mutation. Genes which are regulated by this manner includes *At1g35210*, whose expression is at the same levels in wild-type and *wrky6-3* mutation. Expression of this genes is induced by 4.0-fold by low-B in wild-type, whereas expressions is induced only by 1.2-fold by low-B in *wrky6-3*. Thus, many of the genes induced by low-B are not affected by *wrky6-3* mutation, although inductions of some genes by low-B are inhibited by *wrky6-3* mutation. Annotations of representative genes which are the most strongly regulated by low-B or by *wrky6-3* mutation are listed in table 4-1. Predicted functions of these genes are various.

Discussion

In the present study, I picked up a transcription factor *WRKY6* which affects root elongation under B deficiency. Because B accumulation and *NIP5;1* expression was not altered in the mutant, *WRKY6* affects some other aspect than B accumulation. Promoter activity of this gene is induced near the root tip by B deficiency. To know the role of *WRKY6* in regulation of gene expressions, transcriptome was analyzed in root tips. In addition to the only identified induced gene *NIP5;1* in previous studies, many low-B induced genes were identified, and some of these genes were regulated by *WRKY6*. The most up- or down-regulated genes by low-B or *wrky6-3* mutation are listed (Table 4-1). Ten genes are listed in each four criterion; low-B induction, low-B suppression, *wrky6-3* induction, and *wrky6-3* suppression. Genes listed have diverse functions, showing pleiotropic effects of low-B and *WRKY6* on gene expressions. Inductions of low-B inducible genes are moderately up- or down-regulated by *wrky6-3* mutation.

Among ten most upregulated genes by low-B, expression of *At1g12010* is the most downrgulated by *wrky6-3* mutation. *At1g12010* encodes a putative 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO). Ethylene production is catalyzed by ACO and ACC synthase (ACS) (Yang and Hoffmann 1984), and expressions of genes encoding these enzymes correlate with ethylene production (Rodrigues-Pousada et al. 1993, Gómez-Lim et al. 1993, Gao et al. 2007). Array signals in the present array experiment of twelve ACS homologs and five ACO homologs in *Arabidopsis* are shown (Table 4-2). Expressions of ACSs are slightly upregulated by B deficiency. Expressions of three ACOs are also upregulated by B deficiency and expression of an ACO is slightly downregulated by B deficiency. Restriction by *wrky6-3* mutation is only observed for one ACS and one ACO. Although expression of an ACO homolog is downregulated, these data indicated upregulation of ethylene production in root tip under long-term B deficiency.

The most downregulated gene by *wrky6-3* under low-B is *At1g80530*. This gene is annotated as a nodulin-like protein. There are many genes similar to this gene in *Arabidopsis* and in other plant species. Although an *Arabidopsis* homolog *NFD4* is required for proper nuclear fusion (Portereiko et al. 2006), molecular function of this family is not identified.

There were also genes whose expressions were not up/down-regulated by *wrky6-3* mutation under normal condition and down/up-regulation by low-B is downregulated by *wrky6-3* mutation. Such regulation of gene expressions by *WRKY6* may support root elongation under B deficiency. Although *wrky6-3* mutation affects expression of several low-B induced genes, majority of low-B induced genes are not affected by *wrky6-3* mutation. Additionally, up- or down-regulated genes by *wrky6-3* include transcription factors and ethylene production also seems to be upregulated by B deficiency. A complex mechanism may exist for regulation of gene expressions by B deficiency, and *WRKY6* is only a part of this mechanism. W-box is a binding site for WRKY transcription factors (Eulgem et al. 2000). In the 1.1-kb promoter regions of PR-1 associatedly expressed genes, there are in average 4.3 W-boxes, whereas there are only 1.6

W-boxes in 1.1-kb promoter regions of randomly selected genes (Maleck et al. 2000). Thirty most downregulated genes by *wrky6-3* under B deficiency in the present study were picked up and numbers of W-boxes were counted in 1.1-kb promoter regions. There were only 2.0 W-boxes in average, and only three genes possessed equal to or more than four W-boxes in their 1.1-kb promoter, showing no overrepresentation of W-boxes like another *Arabidopsis* WRKY functioning in regulation of phosphate acquisition and root architecture (Devaiah et al. 2007).

Although many are rest to be clarified in the response of B deficiency and function of *WRKY6* in the response, the first transcription factor which regulates response to B deficiency was identified in this study. Because expression of *NIP5;1* is not regulated by *WRKY6*, there should be more than one pathway to regulate gene expressions in response to B deficiency.

Materials and methods

Plant materials

Arabidopsis thaliana (L.) Heynh. ecotype Col-0 and its mutants and transgenics are used in this study. *wrky6-3* (SALK_12997) and other T-DNA insertion mutants were created by Salk Institute (La Jolla, CA, USA) and obtained from Arabidopsis Biological Resource Center (Ohio State University, USA). Primers were designed as shown in Salk Institute homepage and homozygous line was selected. Other *wrky6* and promoter-GUS lines were provided by Prof. Somssich (Robatzek and Somssich 2001, 2002).

Plant culture conditions

Surface-sterilized seeds were sown on sterilized MGRL growth media (Fujiwara et al. 1992) containing 1% (w/v) sucrose and 0.5% (w/v) gellangum (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. The plates were placed vertically in the growth chamber to allow the roots to grow on the surface of the medium. The concentrations of borate in the culture medium were adjusted by changing the amount of borate added.

qPCR and microarray analysis

Total RNA was extracted and simultaneously treated with DNase using an RNeasy plant mini kit and RNase-free DNase set (Qiagen K.K.) as recommended by the manufacturer. RNA was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and oligo-d$(T)_{16}$ primer and random hexamer. The cDNA was amplified by PCR in a SmartCycler (Cepheid, Sunnyvale, CA, USA) with Ex-Taq R–PCR Version (Takara). Relative amounts of mRNA were calculated using serial dilutions of a concentrated first-strand cDNA stock solution. The primer sets for qPCR were 5'-AGATGATCGAACGGACGTAAA-3' and 5'-CCATTTCGGAAGATTCTCCA-3' (W1 primers) or 5'-ATATTACCGCTGCACGATGG-3' and

5'-ACATTGACCCGGATAGCAAC3' (W2 primers) for *WRKY6*, in additioin to the previously described primers for *NIP5;1* and *elongation factor 1α* (Takano et al., 2005). Microarray analysis was performed using Affymetrix 23k GeneChips following manufacturer's protocol.

Measurement of root-cell length

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

GUS staining

GUS staining was performed overnight as described previously (Shibagaki et al. 2002). Section was counterstained with 0.05% ruthenium red (Sigma-Aldrich).

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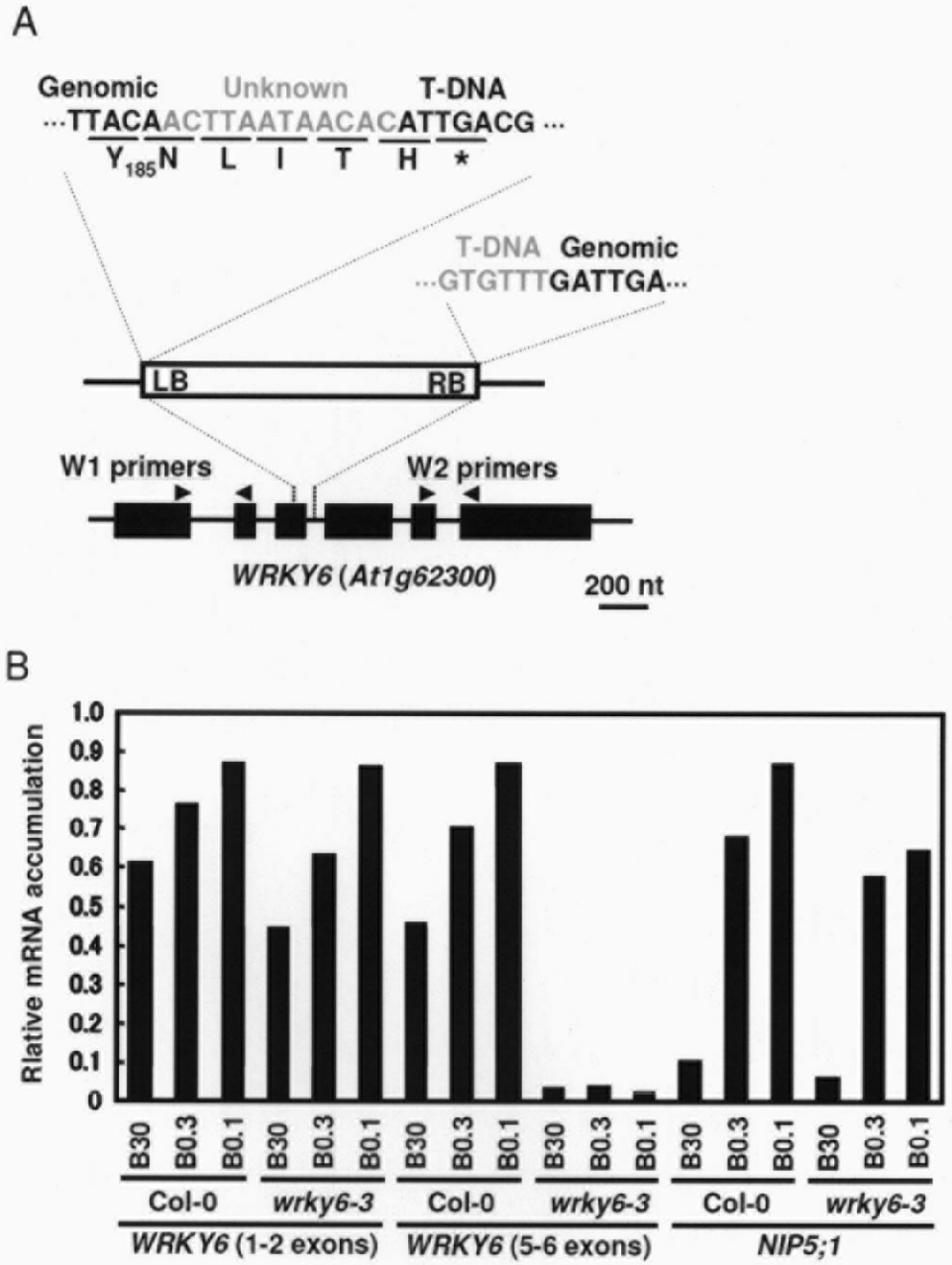

Fig. 4-1. *wrky6-3* mutant. (A) Properties of the T-DNA inserted in *wrky6-3* (SALK_12997). Black boxes represent exons of *WRKY6*. Lines are introns and untranslated regions. Nucleotides in border regions between genomic sequence and T-DNA sequence are shown as well as the corresponding amino acids in left-border margin. Target positions of two sets of primers used for qPCR analysis are indicated by sets of triangles. (B) Quantification of mRNA accumulations of *WRKY6* and *NIP5;1*. Wild-type (Col-0) and *wrky6-3* were cultivated vertically on plates containing 30 μM (B30), 0.3 μM (B0.3) or 0.1 μM (B0.1) borate for ten days. RNA was extracted from whole seedlings and mRNA accumulations of *WRKY6* and *NIP5;1* were measured by qPCR. The first set of primers which amplify *WRKY6* cDNA (W1 primers) target between 1st and 2nd exons of *WRKY6*. The second set of primers which amplify *WRKY6* cDNA (W2 primers) target between 5th and 6th exons of *WRKY6*. Data was standardized with accumulation of *elongation factor 1a* mRNA and the highest data in each primer set (highest value as 0.86).

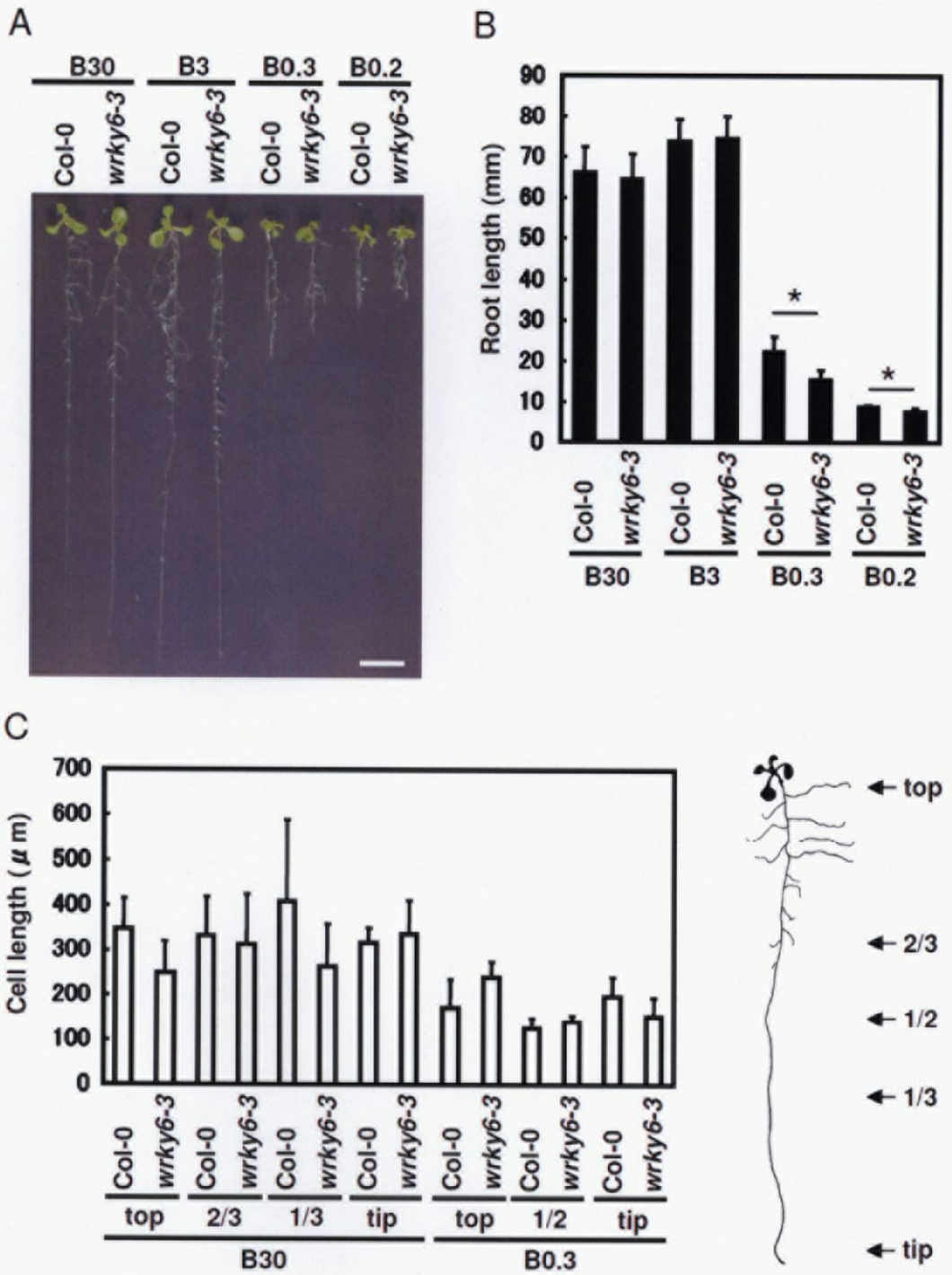

Fig. 4-2. Growth of *wrky6-3* mutant. Difference in growth between wild-type and *wrky6* mutants varied in occasions. Growth of *wrky6-3* when that differed from growth of wild-type is shown. (A) Plants cultivated on plates containing varying concentrations of borate. Wild-type (Col-0) and *wrky6-3* were cultivated for eight days on media containing 30 μM (B30), 3 μM (B3), 0.3 μM (B0.3) or 0.2 μM (B0.2) borate. Bar = 1 cm. (B) Root length of wild-type and *wrky6-3*. Plants were cultivated as in (A) and lengths of main roots were measured. Asterisks indicate statistical difference ($P < 0.05$, $n = 5$). (C) Epidermal cell lengths in main root. Cell lengths were measured at indicated sites in right picture; tip (root-hair zone near the root tip), 1/3 (1/3 from the tip), 1/2 (middle), 2/3 (2/3 from the tip), top (near hypocotyl). There was no significant difference between wild-type and *wrky6-3* ($P > 0.05$, $n = 3$).

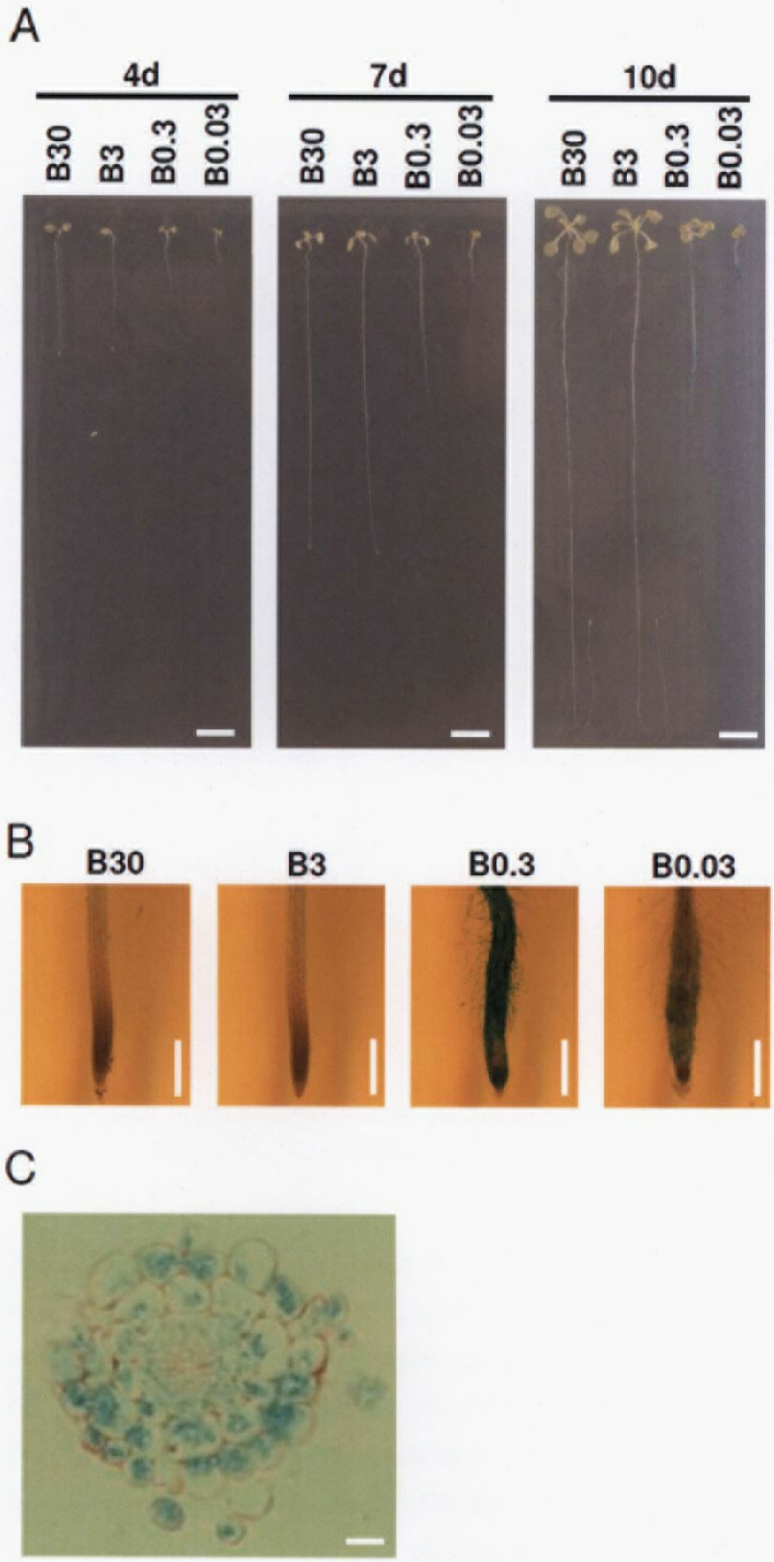

Fig. 4-3. Promoter activity of *WRKY6*. Transgenic plants carrying P_{WRKY6}*GUS* were cultivated on plates containing 30 µM (B30), 3 µM (B3), 0.3 µM (B0.3) or 0.03 µM (B0.03) borate, then stained with X-gluc. (A) Plants were cultivated for 4, 7 or 10 days. Bars = 1 cm. (B) Root tips of 7-d plants. Bars = 500 µm. (C) Cross-section of plant cultivated on medium containing 0.1 µM borate. Sample was prepared at about 1 mm from the root tip. Bar = 10 µm.

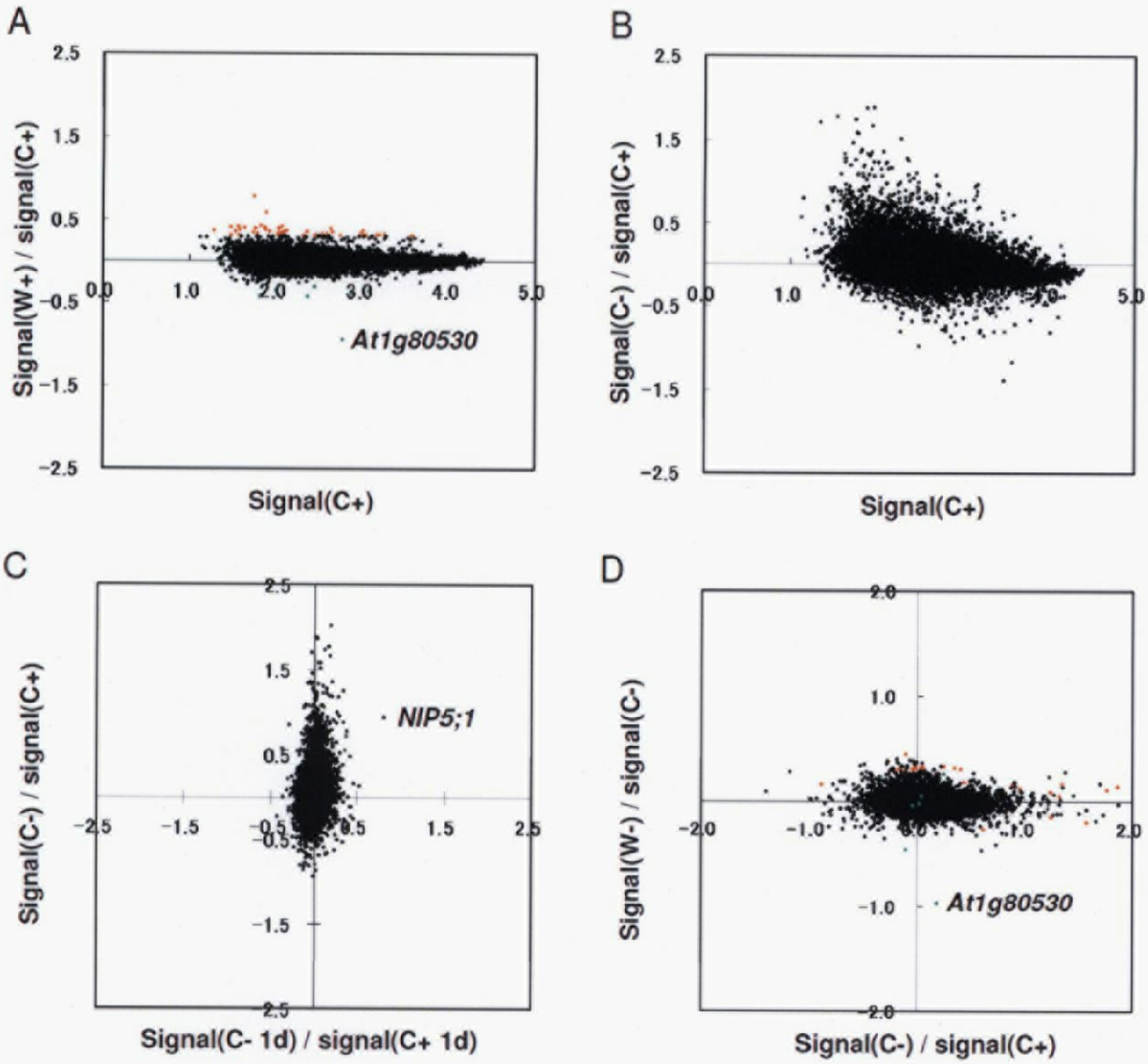

Fig. 4-4. Transcriptome analysis. Wild-type (C) and *wrky6-3* mutant (W) were vertically cultivated for 9 days on media containing 30 μM (+) or 0.1 μM (-) borate. 8-mm root tips were sampled and subjected to transcriptome analysis. Log-ten values of average signals and ratios of average signals are shown. (A) Effect of *wrky6-3* mutation on gene expression under normal condition. Genes induced more than 2-fold are labeled with orange color, and genes suppressed less that 0.5-fold are labeled with green color. (B) Effect of B deficiency. (C) Comparison of inductions between two low-B treatments. *y* value corresponds to the present study, and the *x* value corresponds to a previous study in which plants were hydroponically cultivated for 38 days and treated with normal or low-B culture for 24 hours, and then whole root was subjected transcriptome analysis. (D) Effect of *wrky6-3* mutation on induction by low-B. The same genes are labeled with the same colors in (A).

Table 4-1. Individual gene expressions. Average array signals of two replications in four different samples are shown. C, wild-type; W, *wrky6-3*; +, normal media; -, low-B media. Signal ratios are divisions of average signals. Ten representative genes whose expressions are the most induced or the most suppressed by low-B condition or *wrky6-3* mutation are shown. Annotations of genes are based on web database (ANNOME, http://www.kazusa.or.jp/katana/annome.html).

	Signal				Ratio		
AGI code	C+	W+	C-	W-	C-/C+	W-/C-	Annotation
(Induced by low-B)							
At2g05510	92	144	6857	6382	74.7	0.9	glycine-rich protein
At3g19710	76	179	5574	7711	73.5	1.4	putative BCAA aminotransferase
At5g04950	34	78	2008	2588	58.4	1.3	nicotianamine synthase
At5g09480	56	74	3072	2277	54.5	0.7	PEE-rich protein
At3g45160	22	23	1110	1625	50.4	1.5	unknown
At4g38080	88	86	4038	2636	46.0	0.7	unknown
At1g12010	58	144	2218	1398	38.0	0.6	putative ACC oxidase
At5g58860	60	82	2264	1877	37.5	0.8	cytochrome P450
At5g09530	190	268	6139	4696	32.3	0.8	periaxin-like
At1g73600	52	75	1473	1666	28.5	1.1	phosphoethanolamine N-methyltransferase
(Suppressed by low-B)							
At1g66460	187	157	29	32	0.2	1.1	putative protein
At3g62760	1561	1410	240	263	0.2	1.1	GST
At2g33790	10614	8703	1622	927	0.2	0.6	putative proline-rich protein
At1g09750	1757	1490	243	230	0.1	0.9	unknown
At2g31085	741	790	100	75	0.1	0.8	*CLE6*
At5g62330	1020	2371	133	192	0.1	1.4	putative protein
At3g48940	800	664	93	99	0.1	1.1	remorin-like
At5g15725	310	323	32	33	0.1	1.0	unknown
At5g42600	3836	5232	258	487	0.1	1.9	cycloartenol synthase
At5g04120	3100	2921	125	154	0.0	1.2	phosphoglycerate mutase-like

(Continue to the next page)

Table 4-1 (continued)

AGI code	Signal				Ratio		Annotation
	C+	W+	C-	W-	C-/C+	W-/C-	
(Induced by *wrky6-3* under low-B)							
At5g22555	38	77	30	83	0.8	2.8	unknown
At4g31060	64	92	42	105	0.7	2.5	AP2-EREBP
At4g30810	1835	3487	1567	3806	0.9	2.4	Ser carboxypeptidase II like
At5g38710	98	109	52	126	0.5	2.4	proline oxidase like
At2g37870	123	199	113	270	0.9	2.4	acyl lipid metabolism family
At4g22610	253	366	252	593	1.0	2.4	protease inhibitor
At4g31400	162	224	107	250	0.7	2.3	unknown
At4g30700	112	219	75	174	0.7	2.3	PPR-containing protein
At4g37850	37	94	19	45	0.5	2.3	bHLH
At4g31270	66	105	74	170	1.1	2.3	unknown
(Suppressed by *wrky6-3* under low-B)							
At2g21470	168	194	256	128	1.5	0.5	SUMO activating enzyme 2
At5g51190	138	188	944	465	6.9	0.5	ERF
At4g22710	279	264	593	289	2.1	0.5	cytochrome P450
At3g16530	86	85	307	146	3.6	0.5	putative lectin
At1g12080	6614	5276	2164	994	0.3	0.5	unknown
At5g47450	3799	3296	1896	842	0.5	0.4	*AtTIP2;3*
At1g64160	48	36	262	101	5.4	0.4	dirigent protein, putative
At5g35940	1190	513	940	328	0.8	0.3	putative protein
At1g35210	45	50	179	61	4.0	0.3	unknown
At1g80530	588	66	897	96	1.5	0.1	nodulin-like protein

Table 4-2. Expressions of ACC synthases and ACC oxidases. Average array signals of 12 ACC synthases and 5 ACC oxidases are shown. Ratios represent dividions of average signals. nd: not determined.

	Signal				Ratio	
Gene	C+	W+	C-	W-	C-/C+	W-/C-
(ACC synthase)						
ACS1	nd	nd	nd	nd	nd	nd
ACS2	107	84	nd	nd	nd	nd
ACS3	nd	nd	nd	nd	nd	nd
ACS4	nd	nd	nd	nd	nd	nd
ACS5	72	82	nd	nd	nd	nd
ACS6	289	321	611	333	2.1	0.5
ACS7	176	139	265	245	1.5	0.9
ACS8	nd	nd	nd	nd	nd	nd
ACS9	nd	nd	nd	nd	nd	nd
ACS10	203	181	269	235	1.3	0.9
ACS11	104	135	175	215	1.7	1.2
ACS12	107	89	122	131	1.1	1.1
(ACC oxidase)						
ACO1	297	297	1007	1138	3.4	1.1
ACO2	4368	4279	4822	4605	1.1	1.0
At1g12010	58	144	2218	1398	38.0	0.6
At1g05010	629	832	1763	2223	2.8	1.3
At1g77330	4471	4440	3542	3330	0.8	0.9

Acknowledgements

wrky6-3 (SALK_12997) mutant was provided by Salk Institute and Arabidopsis Biological Resource Center. Promoter-GUS lines and two *wrky* mutants were provided by Prof. Imre E. Somssich. Microarray analysis was performed by Dr. Masami Y. Hirai and RIKEN, and under the support by Ms. Yoko Ide. I would also like to appreciate Mr. Taichiro Iki for support in ethylene quantification.

Chapter 5

Screening of *Arabidopsis thaliana* gain-of-function mutants under nutrient deficiency

Abbreviation

PCR, polymerase chain reaction

Introduction

Screening of loss-of-function mutants like ethylmethanesulfonate-mutagenized mutants in which gene functions are lost by DNA base substitution have been applied to screening of genes functioning in tolerance to nutrient deficiency, although only restricted number of successful screenings are found (ex. Xu et al. 2002). This may partially be because naturally smaller mutants also appear in the screening and it is hard to distinguish sensitive individuals to nutrient deficiency from smaller mutants. We should also be aware that a gene which functions for tolerance to nutrient deficiency does not necessarily improve growth when ectopically overexpressed. For example, we do not find reports describing improvement of growth through overexpressions of *AKT1*, *PHO1*, *IRT1*, and *SULTR1;2* of *Arabidopsis thaliana* which play important roles for nutrient uptake and tolerance to deficiency (Hirsch et al. 1998, Hamburger et al. 2002, Henriques et al. 2002, Varotto et al. 2002, Vert et al. 2002, Yoshimoto et al. 2002, Shibagaki et al. 2002), with exception of *BOR1* which successfully improves tolerance to boron deficiency when ectopically overexpressed by 35S promoter (Noguchi et al. 1997, Takano et al. 2002, Miwa et al. 2006). A direct-forward screening of tolerant mutants to nutrient deficiency will be effective for the purpose of identification of genes which improve plant tolerance to nutrient deficiency.

Screening of gain-of-function mutants may be an effective alternative for isolation of genes which improve plant tolerance to nutrient deficiency. Gain-of-function mutants are such lines in which activities or expressions of endogenous genes are increased by mutations like nucleotide substitutions of T-DNA insertions. There are two types of gain-of-function mutants available as large pools for screening in model plant *Arabidopsis thaliana*. The first is activation-tagged lines in which gene expressions are activated by enhancer sequences inserted in transformed T-DNA (Weigel et al. 2000, Marsch-Martinez et al. 2002, Jeong et al. 2002).

The other is overexpression lines which express cDNAs incorporated into T-DNA under 35S promoter (LeClere and Bartel 2001). FOX lines are especially prepared from even mixture of full-length cDNAs to increase efficiency of screening (Ichikawa et al. 2006). Screening of gain-of-function mutants has identified several genes and a micro RNA which regulate morphology in *Arabidopsis* (for example, Zhao et al. 2001, LeClere and Bartel 2001, Marsch-Martinez et al. 2002, Palatnik et al. 2003, Ichikawa et al. 2006).

In this chapter, activation-tagged lines and FOX lines were screened for mutants which are tolerant to nutrient deficiency. To my knowledge, the present study is the first example of gain-of-function mutants under nutrient deficiency. After extensive screening, however, no obviously tolerant line was isolated. Because the control genes, which improve tolerance to nutrient deficiency, such as *LKS1* and *BOR1* were not recovered in the screening, the present screening is not enough in quantity or in quality. A qualitatively improved screening system is discussed. On the other hand, several larger mutants in size were isolated in the screening and characterized.

Results

Mutant screening

Pools of activation-tagged lines were obtained from stock center. CS21991 consists of the bulk of around 8,000 independent lines and CS31100 consists of the bulk of around 62,000 independent lines. Because the generations of these stocks were not clear, new name for generation, S_1, was created for these stocks because these stocks were the first generation of the screening. Seeds of FOX lines were prepared as bulks of sixty T_2 lines. The total number of lines is around 10,000. Lines without morphological abnormalities were selected for screening. T_2 of FOX lines is also written as S_1.

First screening was performed under various nutrient deficiencies, mainly on plates (Fig. 5-1). Around the half of the sowing was performed by Ms. Mao Sugasawa and Mr. Tsukasa Shigeta. The screening conditions are summarized in table 5-1. Activation-tagged lines were grown hydroponically cultivated with sulfur-deficient media containing 15 μM sulfate, hydroponically cultivated under boron deficiency on rock-wool which was not supplemented with borate, cultivated on boron-deficient media containing 0.03 μM borate, cultivated on nitrogen-deficient media containing 70 μM or 490 μM nitrate, phosphorus-deficient media containing 17.5 μM phosphate, or normally cultivated for twelve days on vermiculite then supplemented with culture containing excessive amount (10 mM) of borate and further cultivated for eight days. FOX lines were cultivated on nitrogen-deficient media containing 70 μM or 490 μM nitrate, potassium-deficient media containing 30 μM potassium ion, sulfur-deficient media containing 15 μM sulfate, or boron-excessive media containing 5 or 6 mM borate. Seeds were sown with the same separations between each other except in the screening

under high boron on soil and screening under low boron by hydroponic culture. This is because different separations cause large variation of plant growth possibly caused by different available amounts of nutrients to each plant. Bigger plants were selected and growth was recovered on soil except in the case of screening of activation-tagged lines with boron-deficient hydroponic culture, in which fertile seeds were gathered. Wild-type plants failed to set seeds under this condition.

In the first screening, around 200 lines were picked up. The following studies were also supported by Ms. Mao Sugasawa. After second round of screenings, nine lines were left for further analysis. Three activation-tagged lines are longer-root mutants under boron deficiency. F21K is a putative low-potassium tolerant mutant. The other five FOX lines are larger lines in size (not tolerant lines to nutrient deficiency). Analysis of three longer-root activation-tagged lines under boron deficiency is described in chapter 6. The names of mutants were designated like 'A119B'. The first character 'A' means that this is an activation-tagged line (or 'F' is for FOX line), 119 is the bulk number, and the last character 'B' means that this line was obtained under boron deficiency (or 'S' is for sulfur deficiency and 'K' is for potassium deficiency).

Characterization of big lines

Several bigger lines under nutrient deficiency were also bigger under normal condition. In these lines, growth ratios between wild-type and mutants were very similar between nutrient deficiency and normal condition, indicating no tolerance to nutrient deficiency attached to these mutants, instead plant size is differentially regulated. The isolated five big lines were designated as F56K, F174K, F178K, F52S, and F174S. Growth of big lines under normal condition is shown (Fig. 5-2). Expansions of rosette leaves are improved in these big lines and timing of flowering was not different. After cultivation for eighteen or nineteen days, fresh weights of whole shoots were significantly improved from 1.5-fold to 2.0-fold in the mutants compared with that of wild-type on the same plate (Fig. 5-2F).

Plant sizes were also observed in the S_3 generations derived from big S_2 segregants of each line. The mutant phenotype (bigger sizes) was observed again only in three lines (F174S, F174K, F178K). Phenotype of the other two lines was not inherited probably because of gene silencing. Growth of S_3 plants of F174S, F174K, and F178K on a normal medium is shown (Fig. 5-3A). In the picture, plants were cultivated for two weeks. Because change in cell ploidy is an endogenous factor which improve plant size (Yoshizumi et al. 2006), cell ploidy was measured in whole shoots of these plants. In plants, there are two mechanisms to duplicate nuclear DNA. The first is duplication in preparation for cell division, and the second is endoreduplication in which DNA is duplicated and cell division does not occur. Cell fates are shifted into endocycle in mature tissues and endoreduplication proceeds up to cells containing 32 sets of chromosomes (32C) or more in *Arabidopsis* (Sugimoto-Shirasu and Roberts 2003). Whole shoots were sampled from two-week old plants and cell ploidy was measured (Fig. 5-3B). There was no difference between wild-type and F174S or F174K. The proportion of 2C cells (not endoreduplicated cells) was slightly decreased in F178K, which was the only significant

difference between wild-type and mutants.

Other phenotypes of big lines

Besides being big, big lines also showed other phenotypes. When big lines were grown on sulfur-deficient media, they accumulated more anthocyanin than wild-type (Fig. 5-4A). When big lines were grown on potassium-deficient media, they were more necrotic than wild-type (Fig. 5-4B). Because big lines grow faster than wild-type, they will be more susceptible to nutrient deficiency and show severer deficiency symptoms than wild-type. This phenotype may be beneficial to discriminate between wild-type and big mutants in segregating populations. When F174S was cultivated on media containing various concentrations of agar, F174S was more tolerant to higher concentrations of agar (the other lines were not tolerant) (Fig. 5-5).

Growth of F21K

F21K is somewhat tolerant to potassium deficiency. Growth of F21K under normal condition and potassium-deficient condition is show in figure 5-6. There was no difference in shoot fresh weights between wild-type and F21K on a normal medium, whereas shoot fresh weight was improved in F21K on a potassium-deficient medium containing 100 μM potassium ion. Under potassium deficiency, expansion of rosette leaves is slightly improved and flowering is faster in F21K.

Discussion

In the present study, screening of gain-of-function mutants was adopted for the first time to isolation of novel genes which improve plant tolerance to nutrient deficiency. Although the screening was performed in a large scale, the screening did not function and no gene to improve tolerance was identified. Two factors may lead to this end. First, there will be only a few endogenous genes which improve plant tolerance to nutrient deficiency, when overexpressed. In *Arabidopsis*, *BOR1* attaches tolerance to boron deficiency and *LKS1* and its two regulators attach tolerance to potassium deficiency (Miwa et al. 2006, Xu et al. 2006). H^+-transporting pyrophosphatase *AVP1* localized to tonoplast attaches tolerance to phosphorus deficiency (Yang et al. 2007). To my knowledge there is no other reported endogenous gene in *Arabidopsis*. This fewness of the endogenous genes which improve tolerance to nutrient deficiency is a handicap for successfulness of the screening. Although, the most critical point is that none of these internal controls was recovered in the screening. This indicates that the screening was far from saturation, in other words, the number of seeds was not enough to assess genome-scale gene functions.

Because there are only a few T-DNA insertions in gain-of-function mutants (Ichikawa et al. 2006), it is hard to complete assessment of all genes in *Arabidopsis* genome with available

gain-of-function mutants with the experiment scale in the present study. A more high-throughput screening with millions of activation-tagged seeds may be successful if we can establish proper screening conditions. Introduction of stronger enhancer in activation-tagged lines will also improve sucreening efficiency. As to FOX lines, position effect may have caused unsuccessfulness of the screening. In some cases in which plant growth is improved by overexpression of plant genes, only lines which are highly expressing the introduced genes show clear improvement of growth under nutrient deficiency (Yanagisawa et al. 2004; Miwa et al. 2006). The same case is also expected for FOX lines in which *Arabidopsis* full-length cDNAs are driven by the same 35S promoter. Introduction of sea urchin insulator sequence upstream of 35S promoter will cancel position effect and improve screening efficiency of FOX lines (Nagaya et al. 2001).

Some endogenous genetic factors are known which improve plant size. Delayed flowering and prolonged vegetative growth in flowering mutants cause bigger rosette leaves in *Arabidopsis* like in *gigantean* mutant. Accelerated endoreducplication also upregulates plant size. In *ilp1-1D* mutant of *Arabidopsis*, endoreduplication is accelerated through activation of a gene encoding a protein homologous to the C-terminal region of mammalian GC binding factor (Yoshizumi et al. 2006). Increased cell ploidy is thought to result in increase of cell size, and then which leads to increased plant size if cell division is not inhibited (Sugimoto-Shirasu and Roberts 2003). There are also exogenous factors. Introduction of *Escherichia coli* glycolate catabolic pathway genes and introduction of an algal cytochrome gene into *Arabidopsis* improve plant growth (Kebeish et al. 2007, Chida et al. 2007). Because the timing of flowering or cell ploidy was not largely different in the isolated three big lines, and only endogenous factors are affected in FOX lines, some unknown mechanism is affected in the analyzed three lines.

Polymerase chain reaction (PCR) products of around 4 kb, 3 kb, 1.8 kb, 2 kb, and 500 bp each were detected in all analyzed S_2 individuals of F21K, F56K, F174K, F52S, and F174S lines each, which amplified a T-DNA region including introduced *Arabidiopsis* full-length cDNAs. Reintroduction of these sequences into *Arabidopsis* may reveal novel mechanisms for plant-size regulation or tolerance to hard gels. Or map-based cloning in phenotype-heritable three lines may be alternative. Tolerance to hard gels may indicate existence of genetic mechanism for compact soils (hard soils) in nature, although further observations are needed. Faster flowering of F21K under potassium deficiency, to my knowledge, is also a novel phenotype.

Materials and methods

Plant materials

Two pools of activation-tagged lines CS21991 and CS31100 of *Arabidopsis thaliana* (L.) Heynh. ecotypes Col-7 and Col-2 each were used in this study with corresponding wild-types. Pool of around 10,000 *Arabidopsis* FOX lines without morphological abnormalities, ecotype Col-0, was

obtained from RIKEN (courtesy of Dr. Takanari Ichikawa and Dr. Minami Matsui). Activation-tagged lines are transformed with activation-tagging vector pSKI015 which possess four repeats of 35S enhancers near the right border of T-DNA (Weigel et al. 2000). FOX lines are transformed with pBIG2113SF which contains various *Arabidopsis* full-length cDNA sequences driven by 35S promoter (Ichikawa et al. 2006).

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 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. 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.

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).

PCR detection of cDNAs

DNA was extracted from leaf as described (Kasajima et al. 2004). T-DNA region containing full-length cDNA of FOX lines was amplified with primer set GS17K18K (5'-GTA CGT ATT TTT ACA ACA ATT ACC AAC AAC-3' / 5'-GGA TTC AAT CTT AAG AAA CTT TAT TGC CAA-3') or primer set AM-F3R2 (5'-CAA CAA CAA ACA ACA ACA ACA TT-3' / 5'-AAG ACC GGC AAC AGG ATT C-3') by Ex Taq (Takara, Kyoto, Japan) in standard PCR cycles. The extension time was prolonged up to 20 minutes when amplification failed.

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Fig. 5-1. An example of the first screening. S_1 seeds of one of the pools of CS31100 were cultivated on a medium containing 70 μM nitrate with the even spacing. Bar = 1 cm (about the real size).

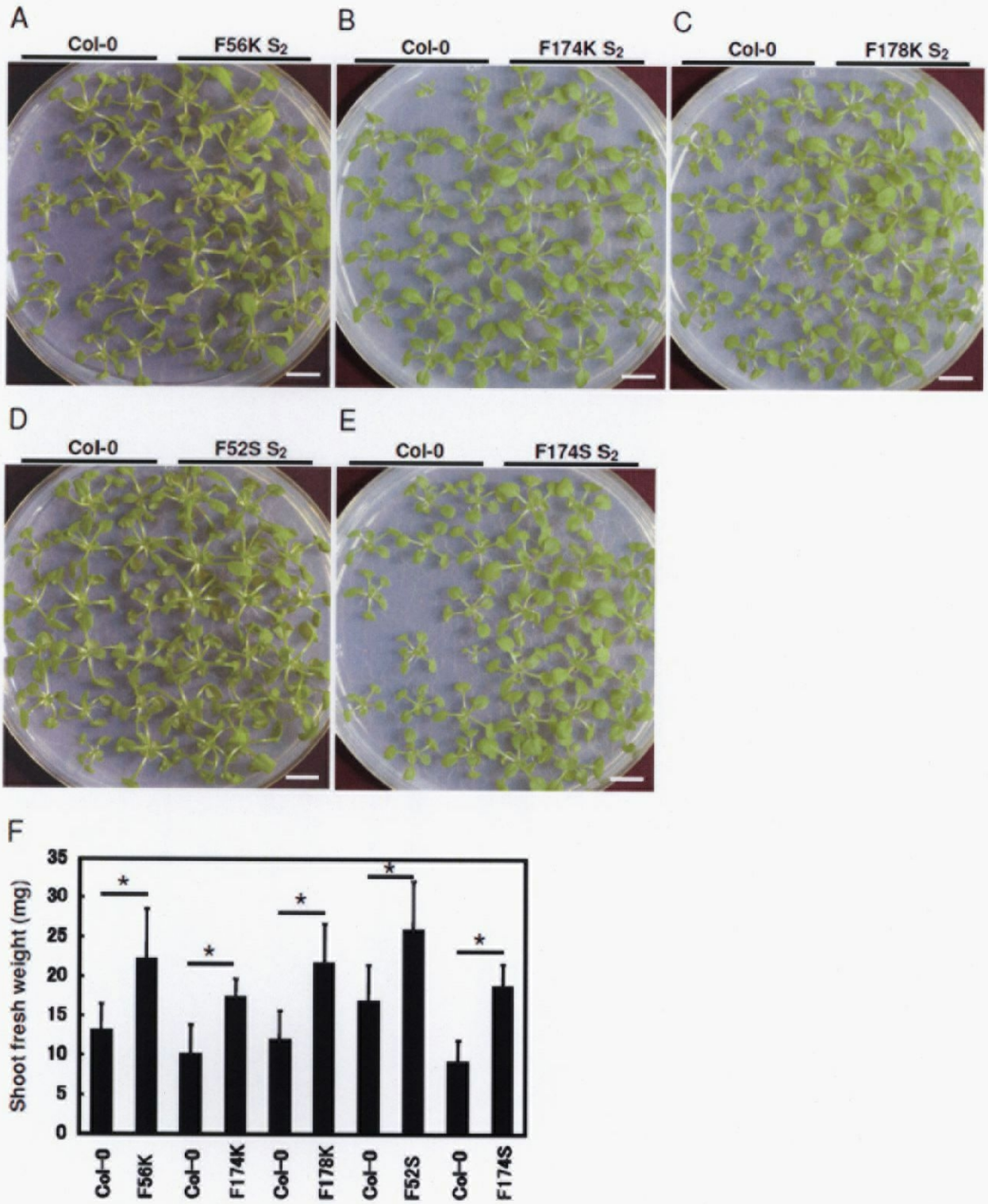

Fig. 5-2. Growth of 'big' lines. Wild-type plants (Col-0) and mutant plants (S_2 progeny) were cultivated for nineteen (A, D) or eighteen (B, C, E) days on standard media containing standard MGRL salt, 1% sucrose, and 1% agar. (A) Growth of F56K. (B) Growth of F174K. (C) Growth of F178K. (D) Growth of F52S. (E) Growth of F174S. Bars = 1 cm. (F) Shoot fresh weights of the plants. Whole shoots were sampled and weighed. n = 14. Asterisks indicate statistical differences between data ($P < 0.05$).

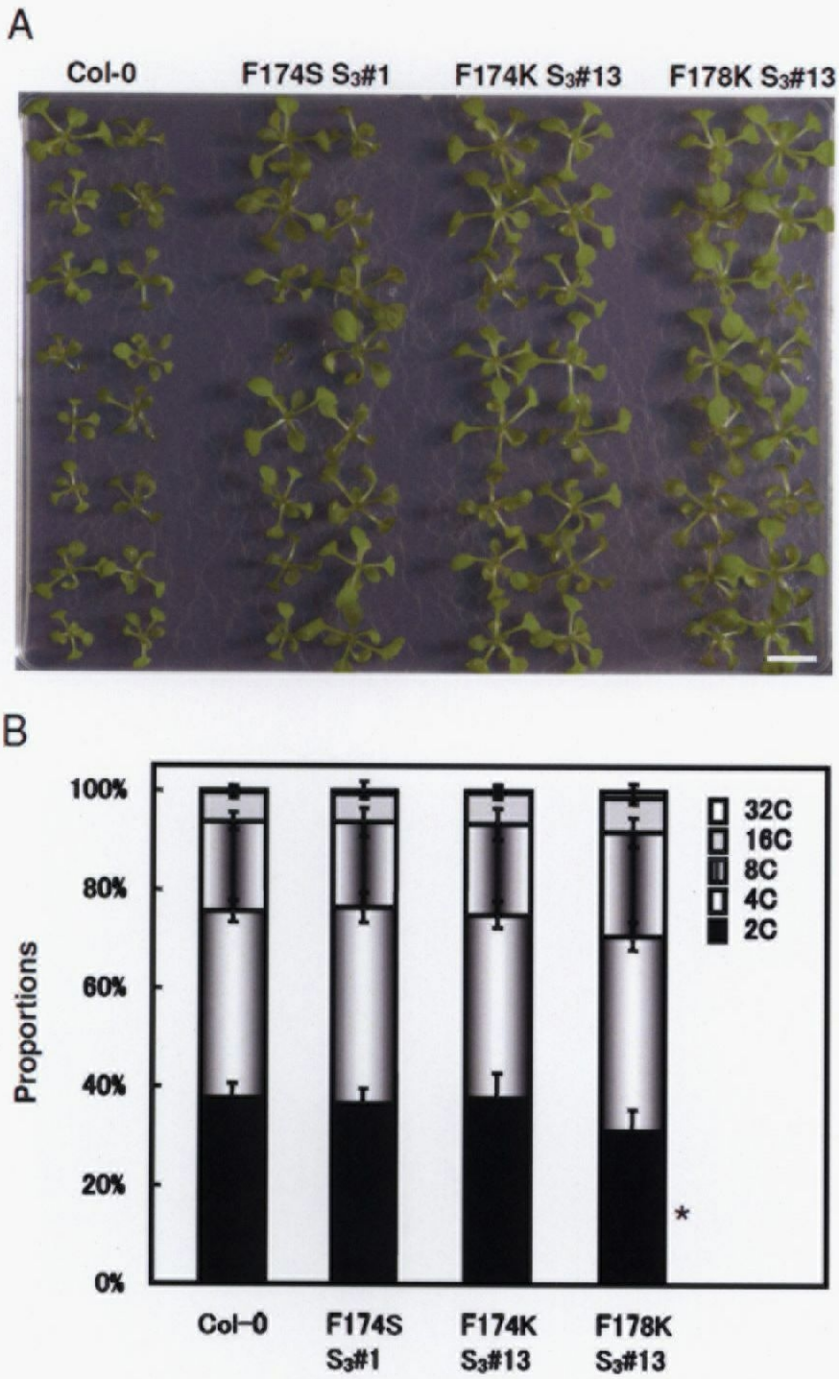

Fig. 5-3. Cell ploidy in the shoot of big lines. Cell ploidy of whole shoot was measured in three big lines whose phenotype was observed in S_3 generation. (A) Wild-type (Col-0) and mutant (S_3 segregants of the indicated pools in the figure) were grown for two weeks on a standard medium. Bar = 1 cm. (B) Proportions of cell ploidy. In total 3,000 cells were measured in the same plants as (A). n = 4. Asterisk indicates statistical difference of mutant from wild-type ($P < 0.05$).

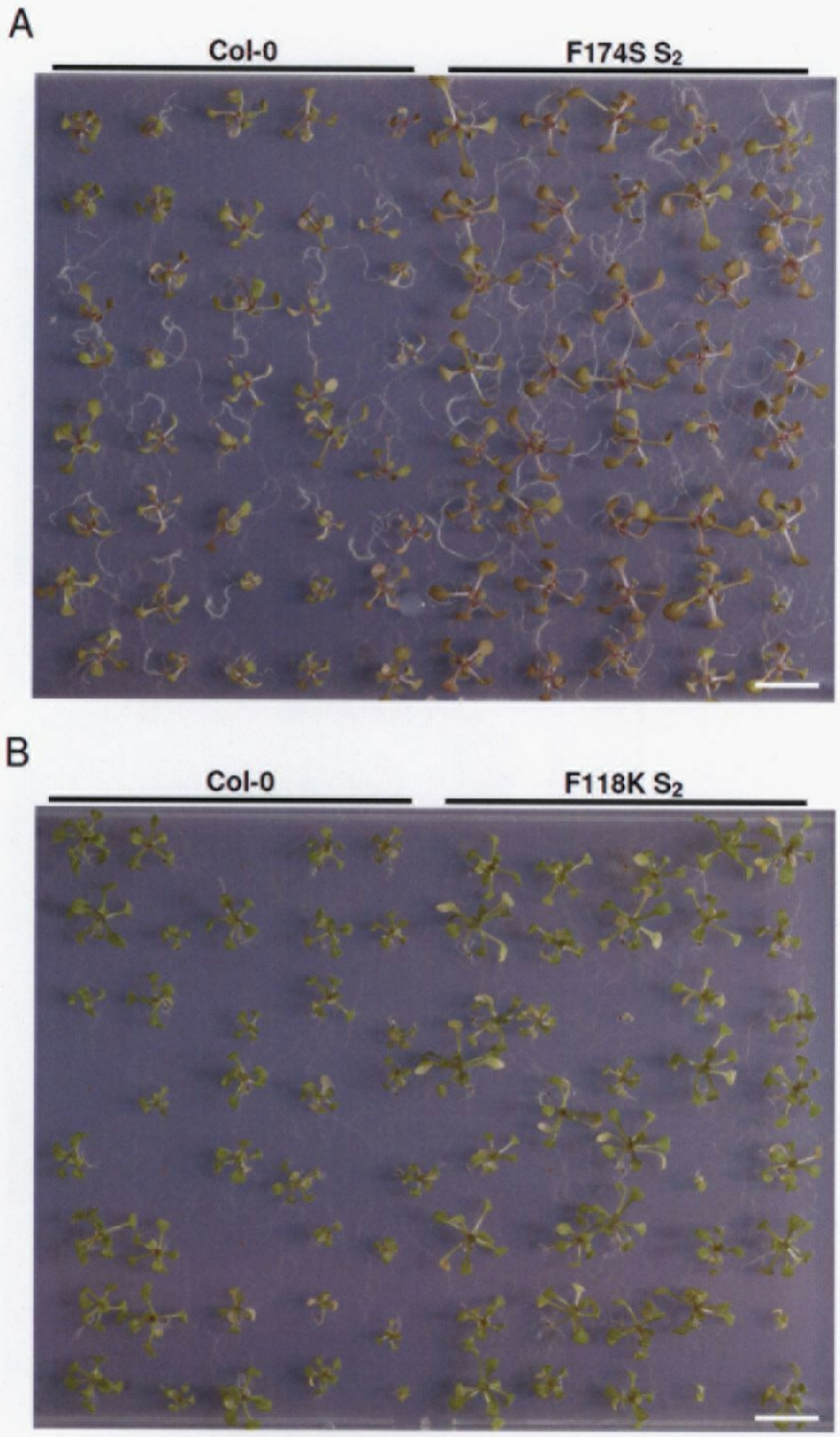

Fig. 5-4. Growth of big lines on nutrient-deficient media. Wild-type (Col-0) and mutants (S_2 generation) were cultivated for two weeks on sulfur-deficient medium containing 15 μM sulfate (A) or potassium-deficient medium containing 100 μM potassium ion (B). The other nutrients were at normal levels in the media. Bars = 1 cm.

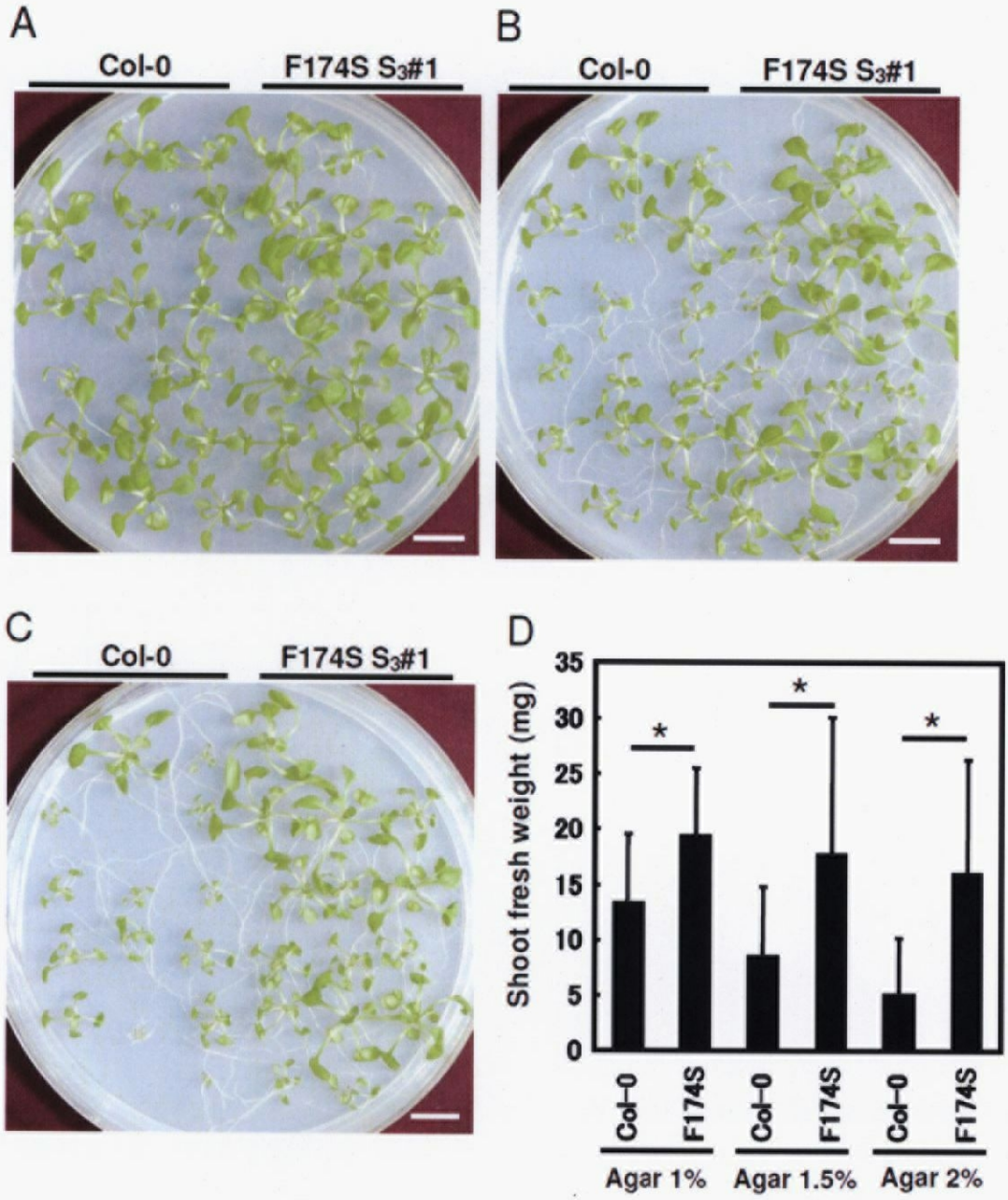

Fig. 5-5. Growth of F174S on media containing various concentrations of agar. Wild-type (Col-0) and F174S mutant (the first pool of the S_3 segregants) were cultivated for eighteen days on media containing 1% (A), 1.5% (B), or 2% (C) agar. Bars = 1 cm. (D) Quantification of shoot fresh weights. Whole shoots were sampled from the same plants as in A, B, and C and weighted. Asterisks indicate statistical difference between data ($P < 0.05$).

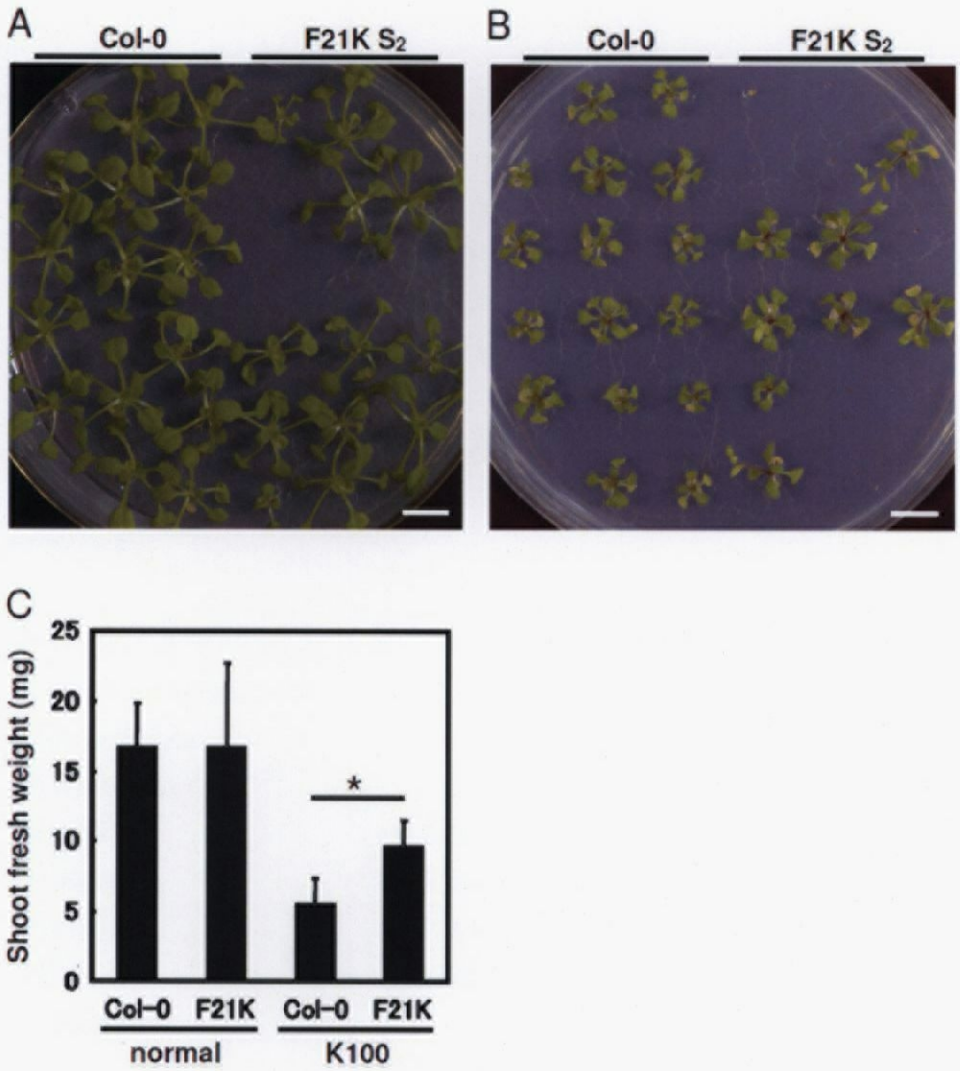

Fig. 5-6. Growth of F21K. Wild-type (Col-0) and F21K mutant (S_2 generation) were grown for nineteen days (A) or eighteen days (B) on normal medium (A) or potassium-deficient medium containing 100 μM potassium ion (B). Bars = 1 cm. (C) Quantification of shoot fresh weights. Whole shoots were sampled from the same plants as in A and B, and weighed. n = 10. Asterisk indicates statistical difference ($P < 0.05$).

Table 5-1. Screening conditions and isolated lines. In the lane 'screening condition', conditions of the screenings are indicated as follows: -, deficiency; ++, toxicity; S, sulfur; B, boron; N, nitrogen; P, phosphorus; K, potassium. Additional characters 'hydroponic' represents screening with hydroponic culture, 'media' represents screening with gel-solidified media, and 'soil' represents screening with vermiculite. Seed stocks were obtained from ARRC (activation-tagged lines CS21991 and CS31100) or RIKEN (FOX lines). 'No. seeds' represents the numbers of the seeds screened under each condition. 'Isolated lines' represents the named of the finally isolated and selected lines in the screening. Three activation-tagged lines are longer-root mutants under boron deficiency. FOX line F21K is a putative low-potassium tolerant line. Other five FOX lines are larger mutants in size (not tolerant lines to nutrient deficiency).

Screening conditions	Seed stock	No. seeds	Isolated lines
(activation-tagged lines)			
-S hydroponic	CS21991	8,000	-
-B hydroponic	CS21991	4,000	-
-B media	CS21991	8,000	A119B, A142B, A152B
-N media	CS31100	115,000	-
-P media	CS31100	48,000	-
++B soil	CS31100	36,000	-
(FOX lines)			
-N media	RIKEN FOX	74,000	-
-K media	RIKEN FOX	60,000	F21K, F56K, F174K, F178K
-S media	RIKEN FOX	60,000	F52S, F174S
++B media	RIKEN FOX	44,000	-

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