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Microarray Note

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

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Abstract Boron (B) is an essential nutrient for plants but is toxic at high levels. We performed transcriptome analysis under conditions of both low and high B in *Arabidopsis thaliana*. There is a positive correlation between the levels of gene induction by low and high B, suggesting that common pathways operate during these responses. Induction by high B was also confirmed for nine genes in an independent experiment. Transcripts of *At1g03770* (transcription factor) and *At5g57340* were found to be elevated in roots, whereas *At2g04040*, *At2g04050*, *At2g04070* (all encoding multidrug and toxic compound extrusion transporters), *At1g32870* (transcription factor), *At5g51440* (heat shock protein-like), *At2g41730* and *At2g21640* are induced in the shoots. The accumulation of both *At2g04050* and *At5g51440* transcripts was further found to be elevated by more than 100-fold after two days of high B treatment. Our findings thus represent the first identification of high B-induced genes.

Key words: *Arabidopsis thaliana*, boron deficiency, boron toxicity, transcriptome analysis.

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 rhamnogalacturonan II complex in the plant cell wall (Matoh et al. 1996). Cross-linking of the rhamnogalacturonan 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, we performed transcriptome analysis of *A. thaliana* in our present study 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 *A. thaliana* 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

Abbreviations: B, boron; MATE, multidrug and toxic compound extrusion; Q-PCR, quantitative polymerase chain reaction; RGI, ratio of gene induction

This article can be found at http://www.jspcmb.jp

subtraction method (Kobayashi et al. 2004). A series of microarray analyses with *A. thaliana* 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 our knowledge, no high B-induced gene has been reported to date.

To further elucidate the overall pattern of gene expression changes and to identify novel genes regulated by the B nutritional status, we performed transcriptome analysis using the following experimental conditions and samples. *A. thaliana* 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 *A. thaliana* 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). We 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 (Q-PCR). 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 1*α were as described previously (Takano et al., 2006). *NIP5;1* expression in *A. thaliana* roots were found to be higher in the RNA samples from low-B treated roots compared with the control roots (kindly examined by Dr. Junpei Takano, data not shown), confirming that our low-B treatments in these particular experiments were at appropriate concentrations. We also measured the B concentration under these culture conditions (Figure 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.

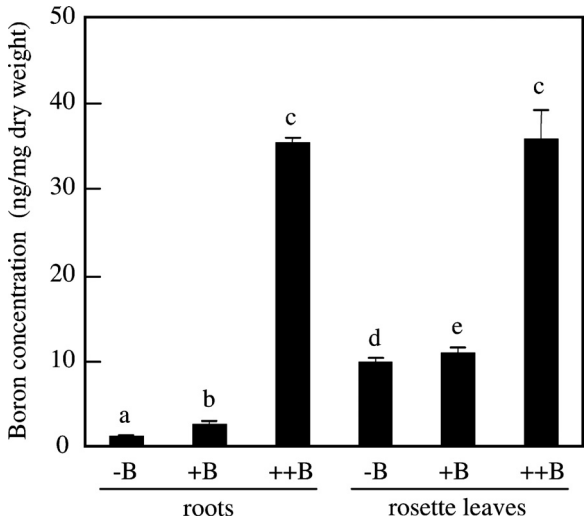

Figure 1. The accumulated levels of B in roots and rosette leaves under various B concentrations in the growth media. *A. thaliana* plants were grown under low-B (−B), control (+B) and high-B (++B) conditions as described in the text. Whole roots and whole rosette leaves were sampled and heat-dried, 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).

Transcriptome analysis was performed with a 23k Affymetrix GeneChip at the Microarray Core Facility in the University of Pennsylvania. We first selected genes which gave a '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 refer to '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 *A. thaliana*, 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 change of mRNA accumulation under low-B or high-B conditions compared with the control, and was calculated for each set of array experiments.

We then compared 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 induction pattern, fold changes in the RGI values between two replications were calculated for each gene. These fold changes were found to be 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 our 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 our present findings is comparable to other transcriptome experiments.

We next compared the RGIs of low- 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 (Figure 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 our replicate experiments, we confirmed the upregulation of mRNA accumulation for a number of key B-responsive genes that had been identified by microarray. We selected genes that satisfied both the following two conditions (formulae 1 and 2):

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

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

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

We 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 nor 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 Q-PCR. Ten of these genes gave reproducible results (data not shown). For the determination of mRNA accumulation for these 10 genes, we 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 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).

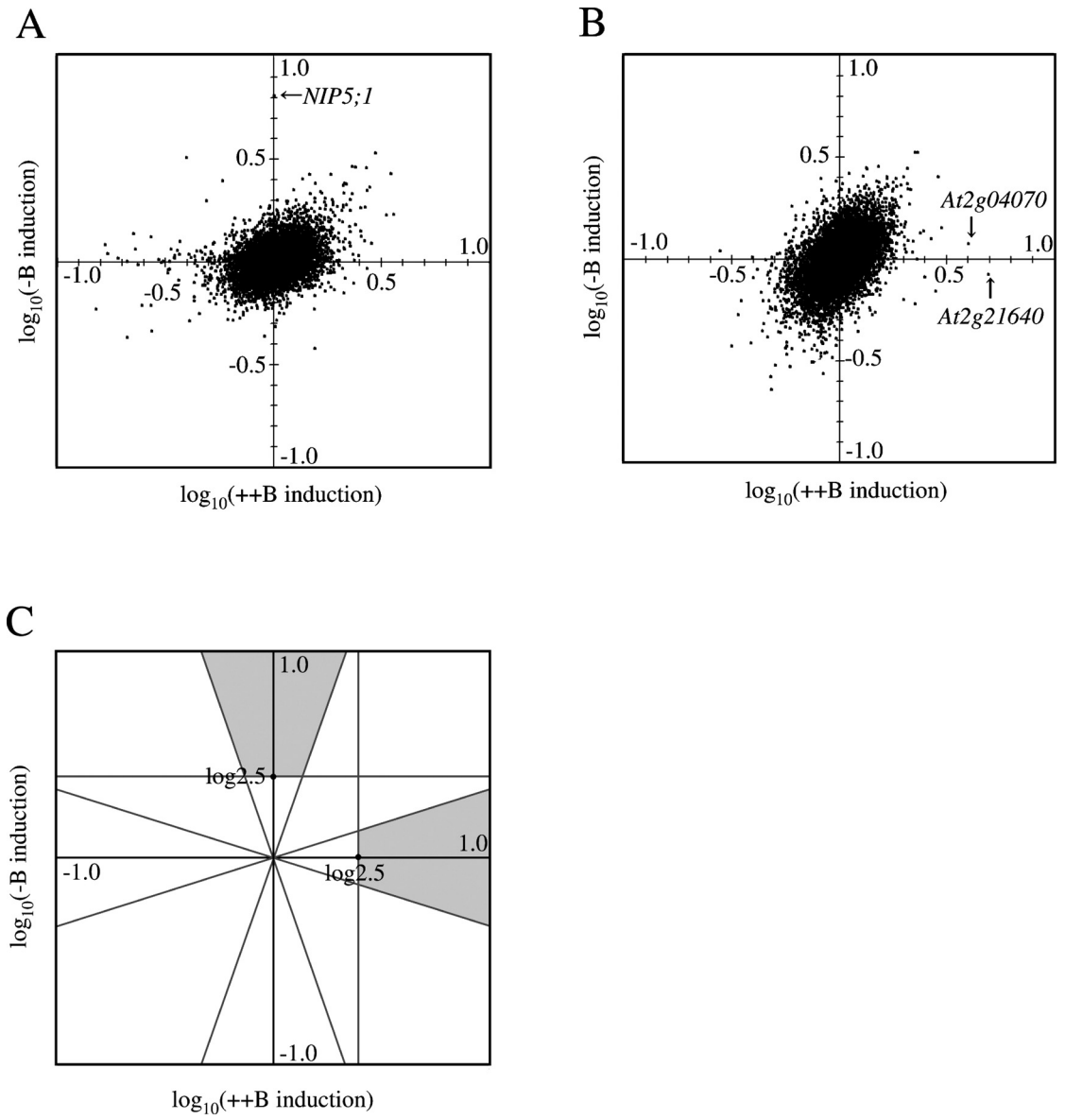

Figure 2. Correlation between the ratios of gene induction following B deficiency and B toxicity. Each spot denotes a single gene, and scatter plots were generated using the log_{10} values of the geometric RGI means of two replicate experiments for high-B (x values) and low-B samples (y values) from the roots (A) and rosette leaves (B) of *A. thaliana*. 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 $x=\log 2.5$, $y=\log 2.5$, $y=-3x$, $y=-1/3\ x$, $y=1/3\ x$, and $y=3x$.

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

We next analyzed the time course of mRNA accumulation for these genes under low-B or high-B

Table 1. Induction of selected *A. thaliana* genes by B nutrition.

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	transcription factor

*P<0.05, versus controls. n=3.

conditions, compared with their expression profiles under control conditions. Wild-type *A. thaliana* 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 Q-PCR. In low-B treated roots (Figure 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

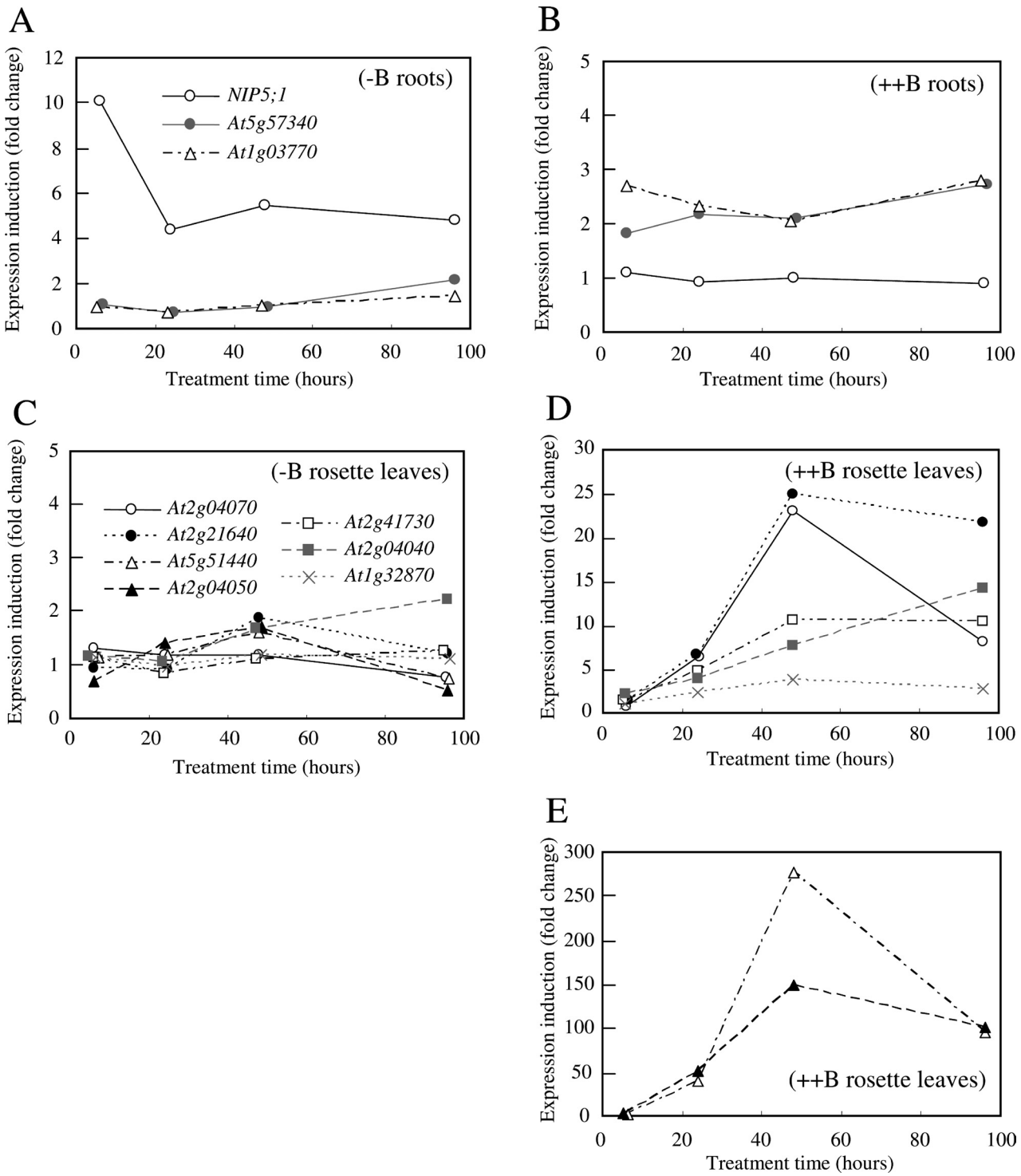

Figure 3. Time-course analysis of mRNA levels of *A. thaliana* genes after exposure to low-B and high-B. *A. thaliana* was hydroponically cultured for 38 days in normal media followed by transfer to either low-B, control or high-B media. Plants were then cultured for a further 6, 24, 48 or 96 hours prior to sampling. mRNA levels were determined by Q-PCR 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).

this induction pattern differs from the findings of a previous report (Takano et al., 2006). Hence, although this analysis was performed only once in our present study, it is possible that subtle differences in our experimental conditions, such as plant density and timing of the sampling, have caused this discrepancy. The expression of *NIP5;1* was found not to alter over four days after high-B treatment (Figure 3B).

The accumulation of *At5g57340* and *At1g03770* transcripts in *A. thaliana* roots occurred after six hours of treatment in high-B medium (Figure 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 (Figure 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 (Figure 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 (Figure 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 our 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, we have identified a number of novel high B-induced genes 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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