Transcriptional Profiling Reveals Adaptive Responses to Boron Deficiency Stress in *Arabidopsis*

Lishun Peng[a,§], Changying Zeng[b,§], Lei Shi[a], Hongmei Cai[a], and Fangsen Xu[a,*]

[a] National Key Laboratory of Crop Genetic Improvement and Microelement Research Centre, Huazhong Agricultural University, Wuhan 430070, China.
E-mail: fangsenxu@mail.hzau.edu.cn

[b] Institute of Tropical Biosciences and Biotechnology (ITBB), State Key Laboratory of Tropical Crop Biotechnology, Chinese Academy of Tropical Agricultural Sciences (CATAS), Haikou 571101, China

* Author for correspondence and reprint requests

Z. Naturforsch. **67 c**, 510–524 (2012); received September 18, 2011/July 11, 2012

Boron (B) is a micronutrient for vascular plants, and B deficiency has been recognized as a limiting factor for crop production in many areas worldwide. To gain a better insight into the adaptability mechanism of plant responses to B starvation, an *Arabidopsis* whole genome Affymetrix GeneChip was used to evaluate global gene expression alterations in response to short- and long-term B deficiency stress. A large number of B deficiency-responsive genes were identified and grouped by their functions. Genes linked to jasmonic acid (JA) showed the most prominent response under B deficiency. The transcripts for biosynthesis and regulation of JA were constantly induced during short- and long-term B deficiency stress. A set of well-known JA-dependent process and responsive genes showed the same expression profile. This suggested that JA could be a pivotal player in the integration of adaptive responses to B deficiency stress. Moreover, other functional groups of B deficiency-responsive genes (including various encoding the biosynthesis of antioxidants, the basic components of Ca^{2+} signalling, protein kinases, cell wall-modifying enzymes and proteins, H^+-ATPase, K^+ transporters, and a set of enzymes involved in central metabolism and cellular growth) were also observed, and their physiological roles under B deficiency stress are discussed. These results provide some information for a better understanding of plant-adaptive responses to B deficiency stress and potential strategies to improve B efficiency in crops.

Key words: Arabidopsis, Boron Deficiency Stress, Transcriptional Profiling

Introduction

Boron (B) is a microelement required for the normal growth of higher plants, and B deficiency has been recognized as an important problem limiting field crop production in many regions of the world, particularly in the high-rainfall regions due to the high solubility of boric acid in water (Shorrocks, 1997). At different growth stages, B-deficient plants usually show a range of symptoms, such as cessation of root elongation, necrosis of meristematic tissues, and loss of apical dominance (Dell and Huang, 1997).

In addition, B deficiency also causes a large number of physiological and biochemical changes. B is considered to be involved in cell wall synthesis and structure, membrane integrity and function, regulation of plasma membrane-bound ATPase and oxido-reductase activities, metabolism of nucleic acids, protein synthesis, hormone metabolism, phenol metabolism, and respiration (for review, see Brown *et al.*, 2002). So far, the only well-defined primary function of B is to maintain cell wall structural and functional integrity through cross-linking two rhamnogalacturonan II (RG-II) monomers (O'Neill *et al.*, 2004), but the plethora of physiological and biochemical effects due to B deficiency cannot be satisfactorily explained by its specific structural role in the cell wall alone. However, it is often considered that most effects in the above list may be secondary or tertiary to the primary functions of B in plants, which may be attributable to the consequences of altering gene expression patterns in response to this nutritional stress. Exploration of the B deficiency-responsive genes would be important to better understand the physiological and biochemical changes due to B deficiency.

§ These authors contributed equally to this paper.

Recently, several successful studies on gene expression in response to B deficiency stress have been reported. In B-deficient tobacco roots, expression of a plasmalemma H^+-ATPase was down-regulated, which resulted in lower nitrate concentrations both in leaves and roots (Camacho-Cristóbal and González-Fontes, 2007). The expression of several genes encoding cell wall-modifying enzymes was down-regulated in B-deficient *Arabidopsis* roots (Camacho-Cristóbal *et al.*, 2008), which could change the extensibility of the cell wall and thus result in cell elongation. Kobayashi *et al.* (2004) found that several reactive oxygen species (ROS)-responsive genes were rapidly induced after 30 min of B deprivation, and Koshiba *et al.* (2009) demonstrated that oxidative damage was the major cause of cell death in B deprived tobacco cells. These studies have provided some valuable insights into B stress responses and their linkage with other biological pathways. However, only a few studies have yet presented a more integrative view of plant responses to B deficiency stress at the transcriptional level. It is unknown what kind of signalling is generated and via which pathway the signal is transmitted, and which down-stream genes expression alters during plant adaptation to B starvation.

In the present study, to gain insights into the mechanism of B deficiency responses of plants, the Affymetrix ATH1 GeneChip was used to assess global changes in gene expression of *Arabidopsis* seedlings in response to short- and long-term B deficiency stress. A set of functional groups of B deficiency-responsive genes were identified in this work. It will help to better understand the adaptive mechanism for plant responses to B starvation

Materials and Methods

Plant materials and growth conditions

A F_8 line (Cs1938) from an *Arabidopsis thaliana Ler*-0 × Col-4 recombinant inbred line (RIL) population developed by Lister and Dean (1993) was used in the experiment, which had been found to be a B-efficient genotype in our previous studies (Zeng *et al.*, 2007, 2008). Seeds of the F_8 line were firstly surface-sterilized with 10% NaOCl (w/v) for 10 min, followed by three rinses with deionized water. After the sterilized seeds had been incubated in the dark at 4 °C for 48 h, they were germinated on gauze fixed between two plastic layers and then floated on quarter-strength Hoagland's solution (Hoagland and Arnon, 1950) that contained 0.324 μM H_3BO_3 (adequate for the normal growth of 10-day-old *Arabidopsis* seedlings). When the *Arabidopsis* seedling roots reached a certain length after 10 d, uniform seedlings were transferred to black cups containing 220 mL of half-strength nutrient solution with different B levels, and one individual plant was retained in each cup for the next two kinds of B stress treatment experiments. All experiments were conducted in an illuminated culture room with the temperature controlled at 25 °C/18 °C (day/night) and a photoperiod of 14 h/10 h (day/night). The solutions were prepared with high-purity water (>18 MΩ/cm conductivity) and renewed every 3 d.

Boron deficiency stress treatments and sample harvest

Two kinds of B deficiency stress experiments were employed in the study: a short-term B deprivation experiment and a long-term B deficiency stress experiment. To eliminate the influence of the circadian clock and plant growth stage effects, all samples (treatments and control) were harvested at the same age of 30 d.

In the short-term B deprivation experiment, the plants of the B-efficient genotype were first cultivated in normal B solution (1.0 μM H_3BO_3) after being transplanted, which was sufficient for maximal biomass accumulation, but did not lead to accumulation of B in plants (data not shown). At 3 h, 24 h, and 72 h before the harvest (30 d), some of those plants were transferred to the B-free solution, and then sampled at the same time like those grown under different B treatment conditions to evaluate gene expression changes after 3 h, 24 h, and 72 h of B deprivation, respectively.

In the long-term B deficiency experiment, the plants were transplanted to low B (0.1 μM H_3BO_3) and normal B (1.0 μM H_3BO_3) solution, respectively, after 10 d of growth in 0.324 μM H_3BO_3 as described above. Then, all of these plants were sampled 20 d after transfer (30 d after sowing).

The two kinds of B deficiency stress experiments were performed simultaneously, so the plants sampled for growing 20 d in normal (1.0 μM H_3BO_3) B solution (refreshed every 3 d) could serve as control for all B treatment (short- and

long-term stresses) samples. Two independent and repeated experiments, including plant culture, stress treatment, and sample harvest, were performed with an interval of two weeks.

RNA preparation and array hybridization

Total RNA from a whole plant for each sample was extracted using TriZol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier's instructions. Probe labelling, chip hybridization, and scanning were performed according to the manufacturer's instructions (http://www.affymetrix.com/support/technical/manuals/expression_manual.affx). RNA from two biological replicates per treatment was hybridized independently to the Affymetrix ATH1 Genome array representing 23,750 *Arabidopsis* genes.

Analysis of GeneChip data

The raw data obtained from the chips were processed using the Affymetrix Microarray Program Suite (MAS 5.0). In order to attain comparable overall intensity between arrays, all arrays were scaled and normalized globally such that their median intensity was equal to a target intensity of 500. Subsequently, MAS 5.0 software was employed to generate signal values (indicating the relative abundance of a particular transcript) and detection call values (indicating the probability that a particular transcript is present or absent). Transcripts designated "absent" in both treated and control samples were removed from subsequent analyses. The gene expression ratios (fold change) between treated and control samples were calculated using the MAS 5.0 software change algorithm. Moreover, a corresponding "change call" ("I", increase; "D", decrease) was also generated by the same software to indicate a significant change. The selected differently expressed genes should comply simultaneously with three criteria: (1) showing a coinciding "change call" in the two biological replicates; (2) fold change from two biological replicates with a standard deviation not exceeding 50% of the mean value; (3) a cutoff value of 2-fold change. MapMan version 3.5.1 (Thimm *et al.*, 2004) was used for analysis of the functional classification and metabolic pathways of B deficiency responsive genes.

Verification of GeneChip data by Northern analysis and real-time quantitative reversed transcription polymerase chain reaction (qRT-PCR)

Northern blot analysis

RNA samples were prepared from the plants treated as described above. Twenty μg total RNA were electrophoresed on an 1% formaldehyde agarose gel and blotted on a Hybond N^+ nylon membrane (BrightStar-Plus, Ambion, Austin, TX, USA) according to the manufacturer's instructions. The filters were pre-hybridized and hybridized with Church buffer [0.5 M Na_3PO_4, 2.5% sodium dodecyl sulfate (SDS)] at 60 °C overnight. Probes were prepared from gel-purified cDNA fragments and labelled with [α^{32}P]dCTP using a random primer (Takara Chemicals, Shiga, Japan). The filters were washed twice in 0.5× saline sodium citrate (SSC) buffer with 0.5% SDS for 5 min at 65 °C and then twice in 0.2× SSC buffer with 0.2% SDS for 5 min at 65 °C. The blots were exposed to an X-ray film for about one week.

Real-time qRT-PCR

Two μg of total RNA (four biological replicates, two same and two different RNA samples from that used in the microarray experiment) were used as template to synthesise single-stranded cDNAs using the M-MLV reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol. Primer Express Software (Applied Biosystems, Foster City, CA, USA) was employed to generate specific primers for further qRT-PCR analysis. The PCR reactions (final volume of 20 μL) were performed with the CFX96™ real-time PCR detection system (Bio-Rad, Hercules, CA, USA), and *ACTIN2* was used as the reference gene. The primer pairs for quantitative RT-PCR and Northern blot analysis are shown in Table I.

Results and Discussion

Morphological effects under different boron deficiency stresses

To analyse the effects of the duration of B deficiency stress, *Arabidopsis* plants were cultivated under short- and long-term B deficiency stress, as described in Materials and Methods. After 3 h and 24 h of short-term stress, no differences in either morphology or biomass were observed between B-deficient plants and control

Table I. Sequences (forward and reverse) of the primers used for quantitative RT-PCR and Northern blot probes.

Gene code	Forward	Reverse
For quantitative RT-PCR		
At1 g19670	TCAAAGCTCACCTACCAACTTC	TGGCCCACGAGTGAGGTGTA
At4 g31800	TGCGTCCCTTCGTATGTCGCTACA	AGAAGGTACAACGCAGCGCAGA
At2 g33790	ATGCGGTGGTGAGACTTGTG	CCGTTCTTGTCCGTCTTTGT
At3 g02850	CGAAACAAACTCGGTAGGAA	GTACTGCAACCGCAAATGTC
At1 g25560	CTCTGCTCAAACTCATCGGCGTAA	TCGGCTTTTCTTGACGCTCATTCT
At1 g11260	CGAAGAAGCCAAAACCAAGC	GGCGACCAAATCGTCAAACT
At5 g44020	TCGTGACTGGAACATCCTGAG	GTTTGGCTAGTCTTCGTGGTG
At1 g32640	GATGAGGAGGTGACGGATACGGAA	CGCTTTACCAGCTAATCCCGCA
At4 g16590	ATGCTTGATGACGAGGATGC	CGACCCTGATTCTTGTTCCA
At1 g74430	CCGGAAGCTCGTGGTTTACATCAA	TAGACCGGCTCTCTTAGGCAGTGA
At3 g18780	TCCCTCAGCACATTCCAGCAGAT	AACGATTCCTGGACCTGCCTCATC
For Northern blot analysis		
At5 g60660	GGTTGCGGTTTCGTCAAAG	GCCAAAGGAGCCAAATGAG
At5 g46900	GATTGCTCTTCTCCTCATCTTC	GATGGAACCTTCTTACCACA
At2 g33790	AGCTCCGATCAAGCTACCAA	CGAGGAAAATCCTGGTTTCA
At4 g15750	CTTCAATATCGACCGTGCTGG	CGGTGGATCAACGTCTTAGCA
At2 g41730	AGTGAAGGTCGTCACCAAGG	GATGGAACCTTCTTACCACA
At3 g18780	CCAGCTCTTCCATCGAGAAG	CCCATTCATAAAACCCCAGC

plants. After 72 h of B deprivation, signs of B deficiency became apparent. These included necrosis of the root tips and dark green colouration of newly emerging leaves (data not shown), together with decreased shoot and root biomass (Fig. 1). Following long-term stress, the symptoms of B deficiency became more obvious, with severely stunted root growth, black dots on the root tips, and dark green and curling leaves (data not shown), as well as significantly reduced shoot and root biomass (Fig. 1).

Gene expression profiles in response to short- and long-term boron deficiency stress

Short-term B deprivation changed the expression of 843 genes significantly, with 446 genes being induced and 397 genes suppressed (Fig. 2A) relative to the control at one time point at least. Among the induced genes, 155, 109, and 216 genes showed increased expression after 3 h, 24 h, and 72 h, respectively. Among the repressed genes, 67, 89, and 323 genes showed decreased expression after 3 h, 24 h, and 72 h, respectively (Fig. 2B). Therefore, although no changes in morphology or biomass were evident after 3 h or 24 h of short-term stress, a set of differentially expressed genes was detected at these time points, and more genes were induced than were suppressed. Goldbach *et al.* (2001) reported that the physical properties of the cell walls of the root tips of squash changed within minutes after B deprivation. This suggested that B deprivation for 3 h and 24 h was sufficient to cause inconspicuous physiological changes in the growing meristematic tissues and consequently evoke a transcriptional response.

The observation that a greater number of suppressed genes was detected after 72 h of B de-

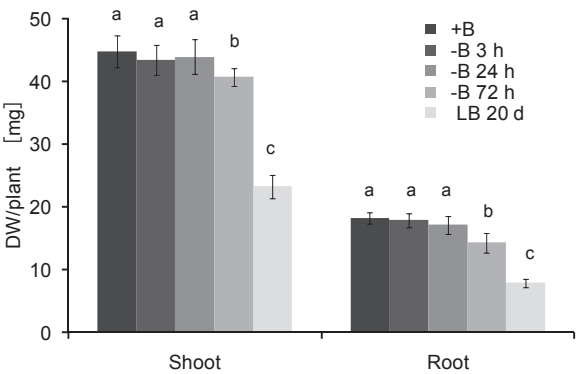

Fig. 1. Shoots and roots dry weight (DW) of 30-day-old *Arabidopsis* plants under different B deficiency stresses and normal conditions. (+B), (-B 3 h), (-B 24 h), (-B 72 h), and (LB 20 d) represent the control, 3 h, 24 h, and 72 h of B starvation, and 20 d of low B stress, respectively. Bars indicate standard errors, $n = 6$. Different letters indicate significant differences at $P < 0.05$.

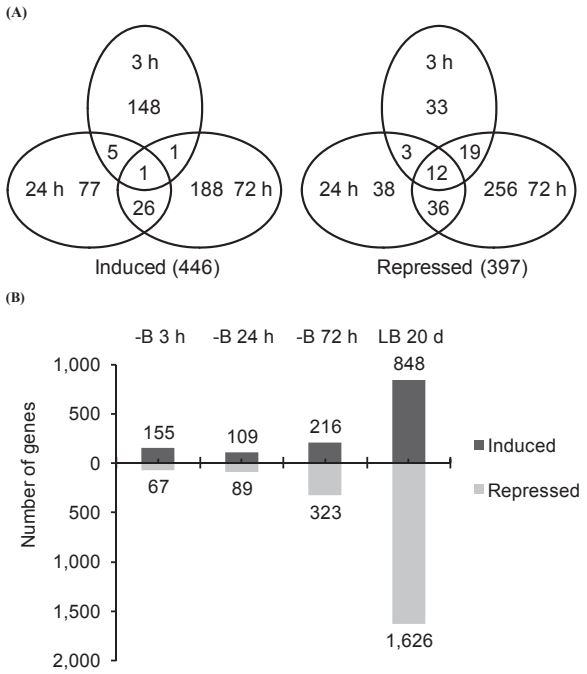

Fig. 2. Number of differentially expressed genes. (A) Venn diagram showing the number of genes induced or repressed at three time points of the short-term B deprivation experiment. (B) Number of genes significantly induced or repressed in response to short-term (3, 24, and 72 h) and long-term (20 d) B deficiency stress experiments.

privation than after B deprivation for 3 h or 24 h might be related to the reduced growth that was observed in response to B deficiency at this time point. Of the 155 genes that were induced after 3 h of B deficiency stress, only six were still induced after 24 h, and only two were still induced after 72 h of B deficiency stress (Fig. 2A). This suggests that most of the genes that were induced after 3 h were affected only transiently by B deficiency. Plants often initiate transient emergency responses to cope with various stresses, and the transient changes may lead to steady, long-term adjustments to adapt to the new environment.

Long-term B deficiency altered the expression of 2,474 genes compared with the control treatment. The observation that the total number of genes that were down-regulated after long-term B deficiency was much higher than the number of up-regulated genes (Fig. 2B) suggests that many different metabolic processes could be affected by long-term B deficiency stress.

Analysis of the source of variability in the array data

To evaluate the reproducibility of the chip data, we determined the correlation between the Affymetrix signals that were generated for the two biological replicates within each treatment. As shown in Table II, correlation coefficients between the two biological replicates varied from 0.953 to 0.990. These results demonstrate the high reliability and reproducibility of the treatments and sampling and technical procedures used.

To confirm the reliability of the chip results, a portion of the data was validated by Northern blot and qRT-PCR analysis. Northern blot analysis was used to verify the different effects of short-term B deficiency stress on the expression patterns of five genes. The Affymetrix chip technique was more sensitive than Northern blot analysis, because transcripts with a relative Affymetrix-normalized signal value of <500 were not detectable by Northern blot analysis under certain conditions (Figs. 3A, B). Nonetheless, in each case, the trend in transcript level (induced or repressed under B deficiency stress) was consistent between the Northern blot and chip analysis (Figs. 3A, B). For the long-term B deficiency stress experiment, RT-PCR was used to confirm the expression level of 10 selected genes. The results of the qRT-PCR analysis were consistent with the chip results (Figs. 3C, D), which indicated that the results obtained with the chip were suitable for further analysis of the expression profiles.

Table II. Correlations of hybridization signal values between two biological replicates in each treatment.

Treatment	Correlation coefficient
+B 1	
+B 2	0.978*
-B 3 h 1	
-B 3 h 2	0.982*
-B 24 h 1	
-B 24 h 2	0.953*
-B 72 h 1	
-B 72 h 2	0.990*
LB 20 d 1	
LB 20 d 2	0.985*

Values between the two replicates were calculated by the Pearson correlation.
* Statistical significance at $P \leq 0.01$.

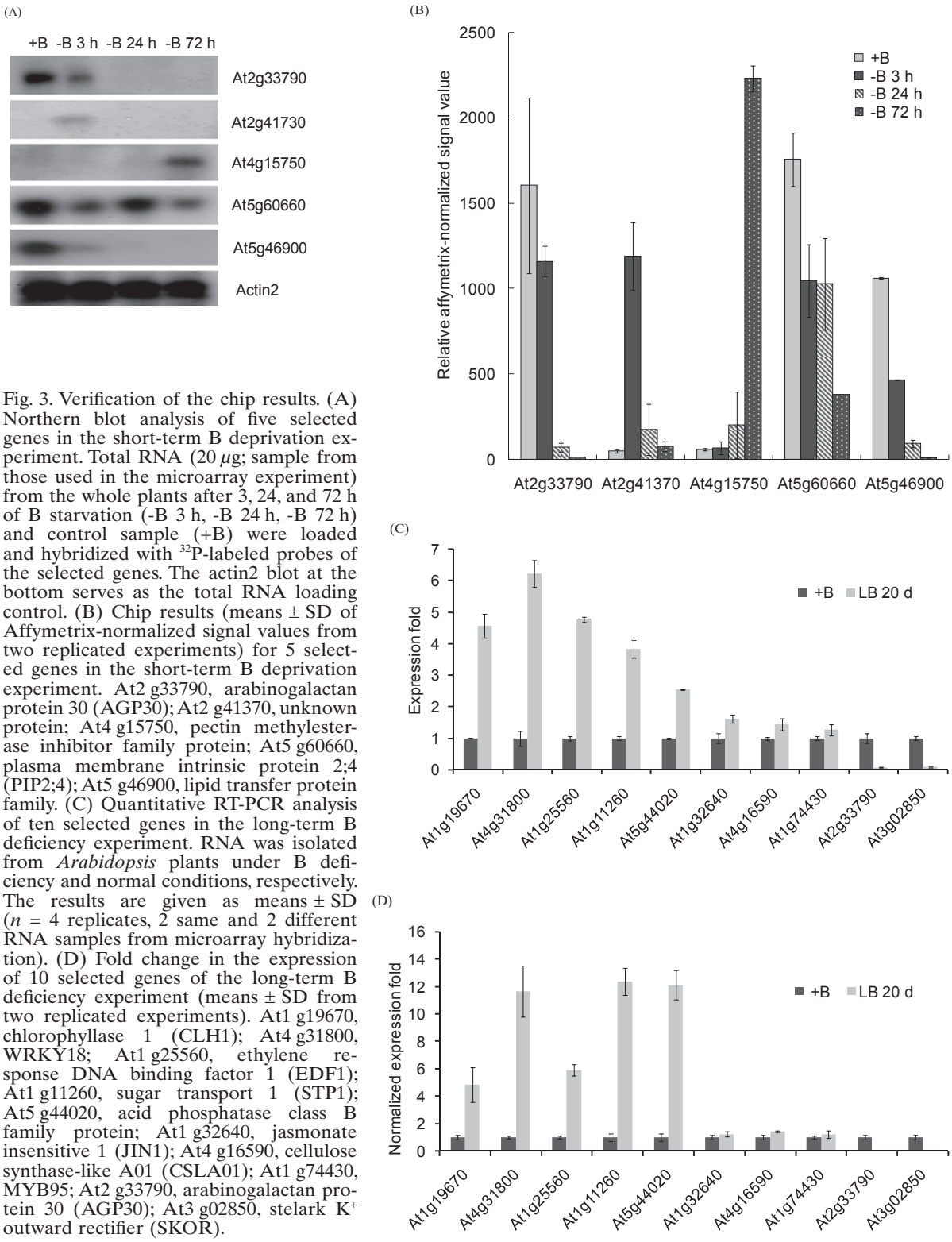

Fig. 3. Verification of the chip results. (A) Northern blot analysis of five selected genes in the short-term B deprivation experiment. Total RNA (20 μg; sample from those used in the microarray experiment) from the whole plants after 3, 24, and 72 h of B starvation (-B 3 h, -B 24 h, -B 72 h) and control sample (+B) were loaded and hybridized with ^{32}P-labeled probes of the selected genes. The actin2 blot at the bottom serves as the total RNA loading control. (B) Chip results (means ± SD of Affymetrix-normalized signal values from two replicated experiments) for 5 selected genes in the short-term B deprivation experiment. At2 g33790, arabinogalactan protein 30 (AGP30); At2 g41370, unknown protein; At4 g15750, pectin methylesterase inhibitor family protein; At5 g60660, plasma membrane intrinsic protein 2;4 (PIP2;4); At5 g46900, lipid transfer protein family. (C) Quantitative RT-PCR analysis of ten selected genes in the long-term B deficiency experiment. RNA was isolated from *Arabidopsis* plants under B deficiency and normal conditions, respectively. The results are given as means ± SD (n = 4 replicates, 2 same and 2 different RNA samples from microarray hybridization). (D) Fold change in the expression of 10 selected genes of the long-term B deficiency experiment (means ± SD from two replicated experiments). At1 g19670, chlorophyllase 1 (CLH1); At4 g31800, WRKY18; At1 g25560, ethylene response DNA binding factor 1 (EDF1); At1 g11260, sugar transport 1 (STP1); At5 g44020, acid phosphatase class B family protein; At1 g32640, jasmonate insensitive 1 (JIN1); At4 g16590, cellulose synthase-like A01 (CSLA01); At1 g74430, MYB95; At2 g33790, arabinogalactan protein 30 (AGP30); At3 g02850, stelark K^+ outward rectifier (SKOR).

Functional analysis of genes responsive to boron deficiency

The genes that were expressed differentially following short-term B deficiency stress were implicated mainly in the regulation of cell wall metabolism, secondary metabolism, stress responses, hormone metabolism, RNA-mediated control of transcription, protein modification and degradation, development, and transport (Fig. 4). Long-term B deficiency was associated with the decreased expression of genes involved in carbohydrate, lipid, and amino acid metabolism, as well as signal transduction, redox regulation, DNA synthesis and repair, RNA synthesis and processing, protein synthesis and targeting, and cell division and the cell cycle (Fig. 4). The presence of co-regulated sets of functionally related genes among the genes that were responsive to B deficiency enabled us to dissect the adaptive responses to B deficiency. The complete list of functional groups of the up- and down-regulated genes is available in the supplementary Table S1, which is accessible at http://www.geboc.org/publication/pls/Table_S1.xls.

Genes related to oxidative stress and detoxification mechanisms

After 3 h of short-term B deficiency stress, the induction of nine glutathione S-transferase (GST) genes by 2- to 19-fold was among the most pronounced changes in gene expression. Another three GST genes were expressed at higher levels than in control plants after 24 h or 72 h of short-term B deficiency stress (Table S1). Members of the GST gene family are frequently induced under various stress conditions and are considered to have a protective role against oxidative stress through their abilities to detoxify metabolites pro-

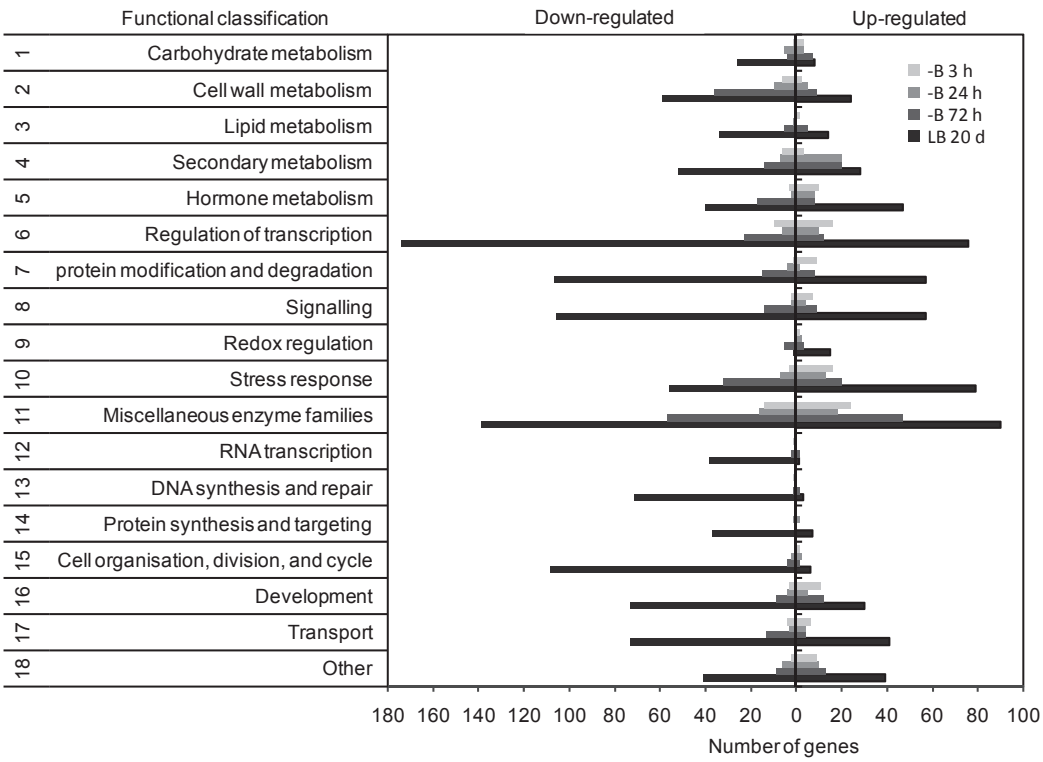

Fig. 4. Functional classification of genes significantly up- or down-regulated in response to short-term (3, 24, and 72 h) and long-term (20 d) B deficiency stress experiments. The charts represent the number of genes in each category that showed at least a 2-fold up- or down-regulation. Genes were classified into functional categories based on established or putative functions as annotated using the MapMan hierarchical ontology (Thimm *et al.*, 2004, http://www.gabi.rzpd.de/projects/MapMan/; Mapping File: Ath_AFFY_TAIR9.m02). Association of individual genes with several functional classes was allowed.

duced during oxidative stress (Wagner *et al.*, 2002). Moreover, three multidrug and toxic compound extrusion (MATE) transporters were up-regulated 2.3- to 10.2-fold at 3 h following B deprivation (Table S1). These MATE transporters are also involved in the detoxification of metabolites of oxidative stress, such as phenols, through sequestration in the vacuole or extrusion at the plasma membrane (Marinova *et al.*, 2007). The results suggest that plants experienced oxidative stress shortly after the perception of B deficiency, and quickly initiated defence responses to cope with this stress.

Several GST and MATE transporter genes were also up-regulated following long-term B deficiency stress (Table S1). Twelve additional genes that encoded a range of ROS-scavenging enzymes were induced 2- to 21-fold. These genes encoded four thioredoxins, five glutaredoxins, one dehydroascorbate reductase, one ascorbate oxidase, and one superoxide dismutase (Table S1). The increased expression of genes that encode various ROS-scavenging enzymes suggests that the redox balance was disturbed by B deficiency, and that more antioxidant enzymes were synthesized to improve the tolerance of the high level of ROS that accumulated in plants subjected to long-term B deficiency stress.

Oxidative damage is the major cause of cell death in B-deprived tobacco cells (Koshiba *et al.*, 2009). Our results underscore the likelihood that oxidative damage is an important consequence of B deficiency stress and that plants have developed a number of antioxidant defence systems to cope with oxidative damage under both short- and long-term B deficiency stress.

Genes related to actions of jasmonic acid and other phytohormones

In the present study, genes linked to the phytohormone jasmonic acid (JA) showed the most prominent response under B deficiency. After 3 h of B deprivation, levels of transcripts that encode allene oxide cyclase 1 (AOC1) and 12-oxophytodienoic acid reductase 2 (OPR2), which are involved in JA biosynthesis, had increased more than 2-fold. Two other genes involved in JA biosynthesis, which encode allene oxide synthase (AOS) and JA carboxyl methyltransferase (JMT), were up-regulated after 24 h of short-term stress. Moreover, the JA-responsive marker genes that encode plant defensin 1.2a (PDF1.2a) and plant defensin 1.2b (PDF1.2b) (Penninckx *et al.*, 1998) were induced 64- and 495-fold after 72 h of short-term stress, respectively (Table S1). This indicates that the JA signalling pathway was activated under short-term B deficiency stress. Owing to the presence of a positive feedback regulatory system in JA biosynthesis (Sasaki *et al.*, 2001), JA signalling is amplified under sustained stress. Accordingly, long-term B-deficiency stress up-regulated the expression of seven genes that encode isoforms of four enzymes involved in JA biosynthesis – lipoxygenase (LOX), AOS, AOC, and OPR (Table S1).

Some well-known JA-responsive genes were also up-regulated by B deficiency. For instance, the genes for seven jasmonate zinc-finger inflorescence meristem (ZIM) domain (JAZ) proteins, two vegetative storage proteins (VSP1 and VSP2), two plant defensins (PDF1.2a and PDF1.2b), two polygalacturonase-inhibiting proteins (PGIP1 and PGIP2), one basic chitinase (CHI-B), and one chlorophyllase (ATCLH1) were induced following either short- or long-term B deficiency (Table S1). The *ATCLH1* gene was the only gene for which levels of its transcripts were elevated significantly at all three time points of the short-term stress and for the long-term stress. Increased expression of *ATCLH1* might protect plants from photo-oxidative damage by degrading the free chlorophyll that is released from thylakoid membranes after tissue damage (Takamiya *et al.*, 2000). The vegetative storage proteins might have no direct defensive functions, because their induction appears to be an adaptation mechanism for the temporary storage of surplus plant resources during various stresses (Staswick, 1994).

Levels of JA often increase following exposure to a number of biotic and abiotic stresses, which is consistent with the role of JA in mediating various defence responses (Creelman and Mullet, 1995). However, this is the first report that genes involved in JA biosynthesis and JA-related defence were activated during short- and long-term B deficiency stresses. We assume that the JA signalling pathway is activated in response to the accumulation of ROS under B deficiency stress. Several sources of oxidative stress stimulate JA accumulation and JA signalling, which are considered to be involved in the containment of lesions that form in response to ROS (Wasternack and Hause, 2002; Overmyer *et al.*, 2000; Rao *et al.*, 2000). JA can activate the ascorbate and glutathione metabolic pathways, which play

important roles in defence responses to oxidative stress (Sasaki-Sekimoto *et al.*, 2005). Several other well-known JA-dependent defence responses, such as anthocyanin biosynthesis and the glucosinolate-myrosinase system, were also activated by B deficiency stress (Table S1). These results indicate that JA could be a pivotal player in the integration of adaptive responses to B deficiency stress. However, it is obvious that JA signaling is not activated uniquely by B deficiency stress, because it is also involved in the adaptation to other nutrient stresses (Nikiforova *et al.*, 2003; Armengaud *et al.*, 2004). It is suggested that changes in gene expression in response to different stresses have both a general component and specific components that are related to a particular stress.

Two genes that encode enzymes involved in the ethylene biosynthesis, aminocyclopropane carboxylate synthase 8 (ACS8) and ethylene-forming enzyme (EFE), and several genes, that encode ethylene-responsive element binding factors (ERF), were also induced during long-term B deficiency stress (Table S1). The ERF1 and ERF2 proteins play an essential role in the integration of the JA and ethylene signal transduction pathways (Pré *et al.*, 2008). Thus, the results suggest that a synergistic relationship exists between the JA and ethylene signal transduction systems in the response to B deficiency stress. Ellis *et al.* (2002) reported that the *Arabidopsis* mutant *cev1* has altered cell walls in the roots due to a reduced cellulose content, and that the JA and ethylene signal pathways are constitutively activated. Moreover, it is well known that B plays a specific structural function in the cell wall, and B deficiency can result in abnormal formation of the cell wall. This indicates that defects in the cell wall could constitutively activate JA- and ethylene-dependent stress and defence responses.

A primary determinant of auxin-mediated plant growth and development is the directional transport of auxin from sites of biosynthesis to sites of action. The observed decrease in the levels of the transcripts that encode the two auxin carriers, pin-formed 1 (PIN1) and pin-formed 2 (PIN2), under conditions of B deficiency (Table S1) might affect plant growth and development by disturbing the polar auxin transport. Furthermore, it has also been proposed that the meristematic regions of B-deficient roots might accumulate excessive levels of endogenous auxin, which inhibits root elongation (Dugger, 1983).

Besides changes in the levels of transcripts implicated in the actions of JA, ethylene, and auxin, long-term B deficiency stress also caused up-regulation of the genes that encode 9-*cis*-epoxycarotenoid dioxygenases 3 and 4 (NCED3 and NCED4). These proteins are isoforms of the enzyme NCED, which is involved in the biosynthesis of abscisic acid (ABA). This is not surprising, given that the phytohormone ABA is a major player in mediating plant adaptation to stress and is induced by a number of abiotic stresses. Long-term B deficiency stress also caused the down-regulation of two genes that are involved in the cytokinin biosynthesis, namely *IPT3* and *IPT5*, which encode adenosine phosphate-isopentenyl transferases (Table S1). Cytokinin regulates cell proliferation and differentiation, and repression of cytokinin biosynthesis might account, at least in part, for the arrest of plant growth under long-term B deficiency stress.

Genes related to secondary metabolism

Anthocyanins are purple pigments that are often considered to be stress indicators. Accordingly, plant leaves often become purple under severe B deficiency condition (Dell and Huang, 1997). In the present study, genes involved in anthocyanin synthesis, such as those that encode anthocyanidin synthase (ANS) and dihydroflavonol 4-reductase (DFR), were up-regulated between 3- and 9-fold under conditions of both short- (24 h and 72 h) and long-term B deficiency stress (Table S1). Moreover, the *UF3GT* and *AACT1* genes, which encode anthocyanin glycosyltransferase and acyltransferase, respectively, and are required for anthocyanidine modification, were up-regulated under long-term B deficiency stress (Table S1). In addition, expression of the gene that encodes the Myb transcription factor named production of anthocyanin pigment 1 (PAP1), which regulates the flavonoid and anthocyanin biosyntheses (Borevitz *et al.*, 2000), was up-regulated in two B deficiency stress experiments (Table S1). Increased rates of anthocyanin synthesis might be an adaptive mechanism to cope with B deficiency. A deficiency of B can make plants more susceptible to damage under high light intensities, owing to light-induced oxidative damage (Cakmak *et al.*, 1995). Anthocyanins can protect plants against photodamage by shielding leaf

tissues from excessive light and scavenging oxygen radicals (Chalker-Scott, 1999).

Another notable functional group of genes involved in the secondary metabolism are those genes that are involved in the glucosinolate-myrosinase system. Glucosinolates have diverse functions. They might be a nutrient sink for nitrogen and sulfur, and the products of their hydrolysis might play important roles in defence against various biotic stresses (Rask *et al.*, 2000). The level of transcripts that encode methylthioalkylmalate synthase 1 (MAM1) increased following exposure to short- and long-term B deficiency stress (Table S1). This enzyme is involved in early steps of glucosinolate biosynthesis from methionine (Kroymann *et al.*, 2001). Two genes that encode cytochrome-P450-dependent mono-oxygenases of the CYP79 family were induced during short- and long-term B deficiency stress (Table S1). These proteins have been implicated in the second step of the glucosinolate biosynthesis by catalyzing the conversion of amino acids to their respective oximes (Hull *et al.*, 2000). The genes that encode three MYB transcription factors, MYB29, MYB51, and MYB76, were also up-regulated (Table S1). These three transcription factors regulate the biosynthesis of indolic and aliphatic glucosinolates (Gigolashvili *et al.*, 2009). Glucosinolates are degradated by myrosinases. Several genes that encode components of different myrosinase complexes were induced by long-term B deficiency stress. These include the genes that encode two myrosinase-like proteins, beta-glucosidase 18 (BGLU18) and beta-glucosidase 26 (BGLU26), two myrosinase-binding proteins, and one myrosinase-associated protein (Table S1).

Genes related to signal transduction and transcription factors

Marked changes in the expression of certain genes that are categorized as regulating signal transduction were also evident during the course of B deficiency stress. In particular, the levels of the transcripts of many genes that participate in calcium ions (Ca^{2+})-mediated signalling mechanisms changed significantly under conditions of B deficiency. These genes included those that encode a Ca^{2+}-binding calmodulin (CAM) and a Ca^{2+}-transporting ATPase (ACA). Genes that encode *ACA12*, *CAM8*, and one calmodulin-related protein were up-regulated after 3 h, and two Ca^{2+}-binding protein genes were up-regulated after 24 h. At 72 h, five genes involved in Ca^{2+}-mediated signalling were up-regulated and three were down-regulated (Table S1). Following long-term B deficiency, eight genes involved in Ca^{2+} signal transduction were up-regulated, and 14 were down-regulated (Table S1). The Ca^{2+} ion is an important cellular signalling component, and transient increases in the levels of cytoplasmic Ca^{2+} are evident in the response to many stresses (Knight *et al.*, 1997). The involvement of Ca^{2+} in the early signalling events that are associated with B deficiency stress was inferred from the observation that changes in the expression of several genes that were responsive to B deficiency were largely repressed by withholding Ca^{2+} from the culture medium or adding a Ca^{2+} channel blocker to B-deficient suspension-cultured tobacco BY-2 cells (Koshiba *et al.*, 2010). The results of the present study support the role of cytoplasmic Ca^{2+} in signal transduction in B-deficient plants.

Mitogen-activated protein kinase (MAPK) cascades are essential signal components in the establishment of resistance in plants against various abiotic and biotic stresses (Pitzschke *et al.*, 2009). Four genes that encode MAPKs – *ATMPK3*, *ATMPK11*, *ATMEK1*, and *ATMKK9* – were induced during long-term B deficiency stress (Table S1). Furthermore, some genes that encode receptor-like kinases (RLKs) were up-regulated during long-term B deficiency stress, including seven genes that encode members of the DUF26 (domain of unknown function 26) family, which are also known as cysteine-rich RLKs (CRKs), and three cell wall-associated kinases (WAKs) (Table S1). The RLKs are also up-regulated by other stresses and are thought to be involved in the regulation of plant defence responses (Czernic *et al.*, 1999). These results suggest that the increased expression of protein kinases could be important components in the signalling of B deficiency stress.

Furthermore, the expression of a large number of genes that encode transcription factors was altered under short- and long-term B deficiency stress. These comprised more than 10 different families, including the apetela 2 (AP2), zinc finger, WRKY, basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), and GATA families (Table S1). Many transcription factors of the WRKY, AP2, and zinc finger protein families are induced or repressed by various stresses (Chen *et al.*, 2002). Previously, only WRKY6 had been reported as a transcription factor involved in the response to B

deficiency. Levels of the *WRKY6* transcript were induced up to two-fold by low B stress, and this induction was essential for normal root growth under low B conditions (Kasajima *et al.*, 2010). In our experiments, expression of *WRKY6* was also up-regulated approximately two-fold after 3 h of short-term B deprivation. No significant change in the levels of the *WRKY6* transcript was observed under long-term stress (data not shown), although 13 genes that encode other WRKY transcription factors were up-regulated during long-term B deficiency (Table S1). These results indicate that the WRKY transcription factors could play key roles in adaptive responses to B limitation.

Genes related to cell wall metabolism

Modulation in the expression of genes that encode various cell wall enzymes and structural proteins was detected mainly after 72 h and during long-term B deficiency stress. The first group of cell wall-related genes that was affected encoded cell wall-modifying enzymes, including polygalacturonases (PGs), pectate lyases, pectin esterases, expansins (EXPs), and xyloglucan endotransglycosylase/hydrolases (XTHs). At 72 h, 14 genes that encode cell wall-modifying enzymes were repressed, and three were induced (Table S1). Under long-term B deficiency stress, 34 genes that encode cell wall-modifying enzymes were repressed, and seven were induced (Table S1). These cell wall-modifying enzymes function in cell elongation and growth by altering the extensibility of the cell wall (Cosgrove, 1999).

Several genes that encode another group of cell wall proteins, the highly glycosylated hydroxyproline-rich arabinogalactan proteins (AGPs) or fasciclin-like AGP, were repressed after 72 h and under long-term B deficiency stress (Table S1). Members of the AGP protein family might play a role in the signal transduction (Schultz *et al.*, 1998). Recently, it has been proposed that alteration of the concentration of B might initiate a cascade of mechanical signals via the cell wall-plasma membrane-cytoskeleton continuum into the cytoplasm, and that this is mediated by conformational changes in membrane-bound proteins. The AGPs might be involved in this process (Goldbach and Wimmer, 2007).

The expression of genes that encode cell wall extensins and extensin-like proteins was also affected by B deficiency. Whereas expression of 10 genes that belong to this class of proteins was down-regulated after 72 h of B deprivation, seven genes were down-regulated after long-term B deficiency stress (Table S1). Extensins and extensin-like proteins are structural components of cell walls and in general are involved in the reinforcement of the cell wall (Sommer-Knudsen *et al.*, 1998). Decreased levels of transcripts that encode extensin and extensin-like proteins during B deficiency stress might affect the cell growth through affecting the physical properties of the cell walls.

Genes related to membrane transport

A deficiency of B disrupts the membrane transport and affects the activities of membrane proteins (Brown *et al.*, 2002). After 24 h of B deprivation, the gene that encodes the stelar K^+ outward rectifier (SKOR), which mediates the delivery of K^+ from stelar cells to the xylem in the roots (Gaymard *et al.*, 1998), was down-regulated 3.7-fold. The level of repression then gradually reached 4.3-fold after 72 h of B deprivation, and was 10.9-fold after long-term stress. Three K^+ transporter genes, *Arabidopsis* K^+ transporter 1 (*AKT1*), *Arabidopsis* K^+ transporter 2 (*AKT2*), and high-affinity K^+ transporter 1 (*HKT1*), were down-regulated under conditions of long-term B deficiency (Table S1). A deficiency of B was reported previously to cause depolarization of plasma membranes and K^+ efflux (Schon *et al.*, 1990). Our results suggest that B deficiency can also affect the expression levels of genes that are related to K^+ transport and thereby affect the membrane transport of K^+.

Under long-term B deficiency stress, the expression of three genes that encode plasma membrane H^+-ATPases (*AHA8*, *AHA6*, and *AHA9*) and two genes that encode vacuolar H^+-ATPases (*VATG3* and *VHA-E2*) were also suppressed about 4- to 24-fold (Table S1). The primary role of H^+-ATPases is to enable secondary transport by generating an electrogenic proton gradient across membranes. According to the acid growth theory, cellular expansion is initiated when the plasma membrane H^+-ATPase acidifies the cell wall. The suppressed expression of the H^+-ATPase genes under conditions of B deficiency could directly affect the transmembrane transport and growth by cell elongation. A previous study reported that B deficiency changed the activity of H^+-ATPase but not the abundance of the protein (Ferrol *et al.*, 1993). In contrast, Camacho-Cristóbal and

González-Fontes (2007) reported results similar to ours insofar as they also found that B deficiency reduced the level of a plasmalemma H^+-ATPase transcript (*PMA2*) in tobacco roots.

Genes related to central metabolism and cellular growth

According to analysis with the software MapMan, transcript levels of genes that encode proteins involved in either the synthesis of macromolecules (DNA, RNA, and protein) or cellular growth (cell organisation, cell division, and cell cycle) were suppressed following long-term B deficiency stress (Table S1). The transcript levels of most of these genes were down-regulated 2- to 3-fold, which suggests that the long-term B deficiency stress resulted in a weak but extensive repression of genes that encode proteins of the central metabolism and cellular growth, in agreement with the arrest of plant growth that was observed under long-term B deficiency stress. Several physiological studies reported a decreased content and synthesis of DNA, RNA, and protein in B-deficient plants, as well as very low mitotic indices (Krueger *et al.*, 1987; Ali and Jarvis, 1988).

Concluding Remarks

We have shown that the expression of several sets of functionally related genes is affected by B deficiency, and discussed the probable physiological roles of these genes under B deficiency stress. Analysis of these functional groups and published information enabled us to establish a comprehensive model of the molecular processes that occur during plant adaptation to B deficiency (Fig. 5).

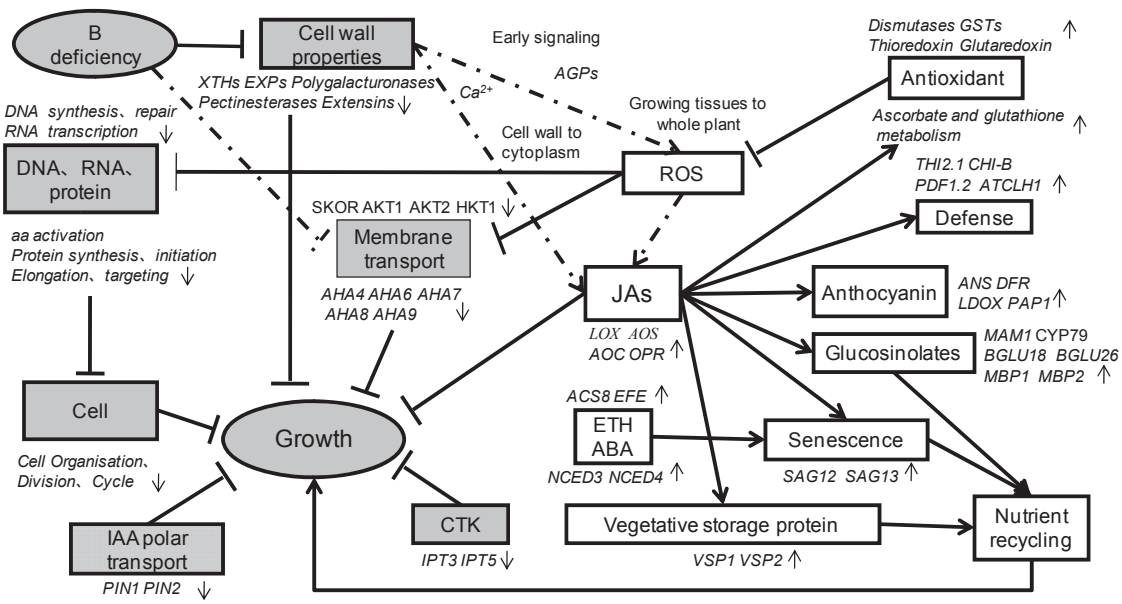

Fig. 5. A model of molecular processes during plant response to B deficiency. Connecting lines between the different events are based on the B deficiency-responsive genes from the present study and published information. Black arrows indicate stimulation, broken lines inhibition. The up and down arrows represent the B deficiency of up- and down-regulated genes, respectively.

Abbreviations: ABA, abscisic acid; ACS, aminocyclopropane carboxylate synthase; AGPs, arabinogalactan proteins; AHA, *Arabidopsis* H^+-ATPase; AKT, *Arabidopsis* K^+ transporter; ANS, anthocyanidin synthase; AOC, allene oxide cyclase; AOS, allene oxide synthase; CHI-B, basic chitinase; ATCLH, chlorophyllase; BGLU, beta-glucosidase; CTK, cytokinin; CYP79, cytochrome *P450 79F*; DFR, dihydroflavonol 4-reductase; EFE, ethylene-forming enzyme; ETH, ethylene; EXP, expansins; GSTs, glutathione S-transferase; HKT, high-affinity K^+ transporter; IAA, indole-3-acetic acid; IPT, isopentenyl transferase; JA, jasmonic acid; LDOX, leucoanthocyanidin dioxygenase; LOX, lipoxygenase; MAM, methylthioalkylmalate synthase; MBP, myrosinase-binding protein; NCED, 9-*cis*-epoxycarotenoid dioxygenase; OPR, 12-oxophytodienoic acid reductase; PAP, production of anthocyanin pigment; PDF1.2, plant defensin 1.2; PIN, pin-formed 1; ROS, reactive oxygen species; SAG, senescence-associated gene; SKOR, stelar K^+ outward rectifier; THI2.1, thionin 2.1; VSP, vegetative storage protein; XTHs, xyloglucan endotransglycosylase/hydrolases.

The model reveals three major categories of plant processes that are affected by B deficiency, namely, signal transduction, defence responses, and cellular growth. Cytoplasmic Ca^{2+}, ROS, JA, and cell wall AGPs were identified as early signalling components in the response to B deficiency, and could play important roles in transduction of the B deficiency signal from the cell wall to the cytoplasm and/or from growing tissues to other plant tissues. Subsequent activation of a set of defence responses plays a crucial role in the containment of ROS-induced lesions and nutrient recycling. Given that most of the defence responses are related to JA, JA could be a main player in the integration of adaptive responses to B deficiency stress. Finally, under long-term B deficiency stress, a large number of transcripts involved in different cellular metabolic processes were repressed coordinately. The majority of the suppressed genes were involved directly or indirectly in cellular elongation growth.

However, this is still only a preliminary analysis of plant responses to B deficiency stress at the whole plant level. Considering that the effects of B deficiency are often initiated in growing meristematic tissues, whole-plant sampling might describe the effects of B deprivation in specific target tissues only inadequately. Moreover, the initial effects of B deficiency occur very rapidly, within minutes, or even less after B deprivation (Goldbach *et al*., 2001). Therefore, the gene expression data that were obtained from the first sampling interval (3 h after B deprivation) define only down-stream reactions to the initial perception of B deficiency. Future research that involves earlier sampling times for the analysis of expression profiles and the sampling of specific tissues (*e.g*., meristematic tissues or cultured cells) should provide further insights into the molecular and cellular responses to B deficiency stress.

Acknowledgements

This work was supported by the National Natural Science Foundation (30971861) and the Fundamental Research Funds for the Central Universities (2011PY150), China.

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