Long-Term Boron-Excess-Induced Alterations of Gene Profiles in Roots of Two Citrus Species Differing in Boron-Tolerance Revealed by cDNA-AFLP

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Boron (B) toxicity is observed in some citrus orchards in China. However, limited data are available on the molecular mechanisms of citrus B-toxicity and B-tolerance. Using cDNA-AFLP, we identified 20 up- and 52 down-regulated genes, and 44 up- and 66 down-regulated genes from excess B-treated Citrus sinensis and Citrus grandis roots, respectively, thereby demonstrating that gene expression profiles were more affected in the latter. In addition, phosphorus and total soluble protein concentrations were lowered only in excess B-treated C. grandis roots. Apparently, C. sinensis had higher B-tolerance than C. grandis. Our results suggested that the following several aspects were responsible for the difference in the B-tolerance between the two citrus species including: (a) B-excess induced Root Hair Defective 3 expression in C. sinensis roots, and repressed villin4 expression in C. grandis roots; accordingly, root growth was less inhibited by B-excess in the former; (b) antioxidant systems were impaired in excess B-treated C. grandis roots, hence accelerating root senescence; (c) genes related to Ca2+ signals were inhibited (induced) by B-excess in C. grandis (C. sinensis) roots. B-excess-responsive genes related to energy (i.e., alternative oxidase and cytochrome P450), lipid (i.e., Glycerol-3-phosphate acyltransferase 9 and citrus dioxygenase), and nucleic acid (i.e., HDA19, histone 4, and ribonucleotide reductase RNR1 like protein) metabolisms also possibly accounted for the difference in the B-tolerance between the two citrus species. These data increased our understanding of the mechanisms on citrus B-toxicity and B-tolerance at transcriptional level.

Keywords: boron-excess, cDNA-AFLP, Citrus grandis, Citrus sinensis, roots
**INTRODUCTION**

Boron (B), an essential element for normal growth and development of higher plants, is taken up by roots in the form of boric acid from soil solution. Despite this, B is toxic to higher plants when present in excess (Nable et al., 1997; Chen et al., 2012; Sang et al., 2015). Although B-toxicity is less common than B-deficiency, it is still a serious problem in many agricultural regions across the world. However, the molecular mechanisms on B-toxicity and B-tolerance in higher plants are not fully understood.

In higher plants, B-excess cause impairments of cellular functions and physiological and biochemical processes, such as plant growth (Ayvaz et al., 2012; Guo et al., 2014), hormone balance (Ayvaz et al., 2012, 2015), uptake and use efficiency of other mineral elements (Papadakis et al., 2003; Aquea et al., 2012), leaf photosynthetic electron transport chain (Han et al., 2009), photosynthetic rate (Han et al., 2009; Sheng et al., 2010; Chen et al., 2012; Guo et al., 2014), photosynthetic enzyme activities (Han et al., 2009), reactive oxygen metabolism (Keles et al., 2004; Cervilla et al., 2007; Han et al., 2009), chlorophyll (Chl) and carotenoid (Car) levels (Han et al., 2009; Ayvaz et al., 2012), cell wall biosynthesis (Huang et al., 2016); carbohydrate (Keles et al., 2004; Papadakis et al., 2004a,b; Han et al., 2009), energy (Reid et al., 2004; Guo et al., 2014), nucleic acid (Wang et al., 1995), and nitrogen (N; Cervilla et al., 2009; Chen et al., 2014) metabolisms, and leaf and root structure (Papadakis et al., 2004a,b; Chen et al., 2012; Huang et al., 2014; Mesquita et al., 2016).

Transcriptomic analysis not only offers us the chance to elucidate the molecular mechanisms on plant B-toxicity and B-tolerance but also is very useful for us to obtain B-excess-responsive genes possibly responsible for B-tolerance. There are several studies investigating B-excess-induced alterations of gene profiles in higher plants. Using microarray, Kasajima and Fujiwara (2007) isolated many high-B-induced genes from Arabidopsis thaliana roots and rosette leaves; nine of which were validated in an independent experiment. Using suppression subtractive hybridization (SSH), Hassan et al. (2010) found 111 up-regulated clones showing similar to known proteins from high-B-treated roots of the B-tolerant barely bulk, thereby suggesting that both polyamines and ascorbate-glutathione pathway played a role in the B-tolerance of barely. Padmanabhan et al. (2012) used SSH to isolate 219 and 113 unique B-excess-responsive non-redundant expressed sequence tags (ESTs) from extremely B-tolerant Puccinellia distans and moderately B-tolerant Gypsophila arrostil plants, respectively. In addition to restricting B accumulation in plant tissues via enhancing the expression of efflux transporters similar to barely Bot1, genes related to stress/defense, protein synthesis, cellular organization, and signal transduction had a positive role in the B-tolerance of P. distans. Aquea et al. (2012) obtained 240 up-regulated genes, which mainly function in ABA signaling, ABA response, or cell wall modifications, and 211 down-regulated genes, which are mainly related to glucosinolate biosynthesis, water, or nutrient transport, suggesting that B-toxicity-induced water stress was responsible for root growth inhibition under B-stress. Recently, Tombuloglu et al. (2015) used high-throughput RNA-Seq to examine B-toxicity-induced alterations of gene profiles in barely and observed a total of 14,385 and 11,472 differentially expressed transcripts from high-B-treated roots and leaves, respectively, and concluded that genes involved in cell wall and cytoskeleton, plasma membrane, stress response, and signal transduction played key roles in the B-tolerance of barely. Although the transcriptional responses of herbaceous plants to B-toxicity have been investigated in some details, such data are very limited in woody plants.

In China, B-excess is observed in some citrus orchards. Li et al. (2015) measured the water-soluble B content of 319 soil samples and the B concentration of 319 leaf samples from 319 pummelo (Citrus grandis) orchards located in Pinghe, Zhangzhou, China. Up to 22.9 and 74.8% of orchards were excess in soil water-soluble B and leaf B, respectively. Therefore, it is very important to understand the molecular mechanisms on B-toxicity and B-tolerance of citrus. cDNA-amplified fragment length polymorphism (cDNA-AFLP), which does not need prior sequence information for the amplification, is a highly sensitive and reproducible approach for detecting polymorphisms in DNA. Therefore, cDNA-AFLP has been widely used for the identification of various stress-regulated genes and for the isolation of novel genes (Ditt et al., 2001; Fusco et al., 2005; Craciun et al., 2006; Paun and Schönswetter, 2012; Zhou et al., 2013; Vantini et al., 2015). Previously, we used cDNA-AFLP to assay long-term B-excess-responsive genes in B-tolerant Citrus sinensis and B-intolerant C. grandis leaves and obtained 132 and 68 differentially expressed genes from B-toxic C. grandis and C. sinensis leaves, respectively. Subsequent analysis suggested that the higher mRNA levels of genes involved in photosynthesis in B-toxic C. sinensis leaves were responsible for the higher CO₂ assimilation, thus contributing to the B-tolerance of C. sinensis. Some genes related to stress responses also played a role in the B-tolerance of C. sinensis (Guo et al., 2014). In this study, we further examined long-term B-excess-induced alterations of gene profiles revealed by cDNA-AFLP in B-tolerant C. sinensis and B-intolerant C. grandis roots (Guo et al., 2014; Huang et al., 2014; Sang et al., 2015). The objectives were (a) to determine the differences in B-excess-responsive genes between roots and leaves; (b) to explore the mechanisms on B-toxicity and B-tolerance at transcriptional level; and (c) to obtain B-excess-responsive genes possibly contributing to the B-tolerance of C. sinensis.

**MATERIALS AND METHODS**

**Plant Materials and Experimental Design**

Boron-tolerant “Xuegan” (C. sinensis) and B-intolerant “Sour pummmel” (C. grandis) seedlings were used in this study. Both seedling culture and B treatments were performed according to Guo et al. (2014). In a word, 13-week-old seedlings grown in 6 L pots containing sand in a greenhouse under natural photoperiod at Fujian Agriculture and Forestry University (FAFU), Fuzhou, China (26°5’ N, 119°14’ E), were irrigated every 2 days until dripping with nutrient solution containing 6 mM KNO₃, 2
mM NH$_4$H$_2$PO$_4$, 4 mM Ca(NO$_3$)$_2$, 1 mM MgSO$_4$, 10 µM H$_2$BO$_3$, 2 µM ZnSO$_4$, 2 µM MnCl$_2$, 0.5 µM CuSO$_4$, 0.065 µM (NH$_4$)$_6$Mo$_7$O$_24$, 20 µM Fe-EDTA, and 10 µM (control) or 400 µM (B-excess) H$_2$BO$_3$ for 15 weeks. Thereafter, about 5-mm long root tips from new white roots were excised and immediately immersed in liquid nitrogen, and then stored at −80°C until being used for the measurements of total soluble proteins and enzyme activities, and cDNA-AFLP and qRT-PCR analysis. The remaining seedlings were used to assay root B and phosphorus (P) concentrations.

**Measurements of Root Total Soluble Protein, B, and P Concentrations**

Root concentration of total soluble proteins was assayed according to Bradford (1976) after being extracted with 50 mM Na$_2$HPO$_4$–KH$_2$PO$_4$ (pH 7.0) and 5% (w/v) insoluble polyvinylpyrrolidone. There were five replicates per treatments.

For the measurements of root B and P concentrations, fibrous roots were harvested and dried at 70°C. Root B concentration was determined by ICP emission spectrometry after microwave digestion with HNO$_3$ (Wang et al., 2006). Root P concentration was determined according to Ames (1966). There were four replicates per treatments.

**RNA Extraction, cDNA Synthesis, and cDNA-AFLP Analysis**

Equal amounts of frozen roots from five excess B-treated or control seedlings (one seedling per pot) of *C. grandis* or *C. sinensis* were mixed as a biological replicate. There were three biological replicates for each B treatment. Total RNA was extracted from ca. 300 mg of frozen roots using Recalcitrant Plant Total RNA Extraction Kit (Centrifugal column type, Biotek Corporation, China). cDNA synthesis and cDNA-AFLP analysis were performed according to Guo et al. (2014) and Lu et al. (2015). Database resources of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) were used for the blast analysis of the differentially expressed transcript-derived fragments (TDFs) from excess B-treated *C. grandis* and *C. sinensis* roots. TDFs were blasted using BLASTX and BLASTN search engines (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and a TDF was considered to be homologous when it had an *E* < 0.001 and a similarity ≥50%. Their functional categories were assigned based on the Gene Ontology (http://amigo1.geneontology.org/cgi-bin/amigo/go.cgi) and UniProt (http://www.uniprot.org/) database.

**qRT-PCR Analysis**

Root total RNA was extracted as described above. qRT-PCR analysis was carried out according to Zhou et al. (2013) and Lu et al. (2015). The sequences of the specific F and R primers designed from the sequences of 15 singleton TDFs using Primer Premier Version 5.0 (PREMIER Biosoft International, CA, USA) were summarized in Table S1. The samples subjected to qRT-PCR were performed in three biological replicates with two technical replicates. Each biological replicate was created by pooling equal roots from five seedlings (one seedling per pot). *Citrus actin* (GU911361.1) was used as an internal standard for

the normalization of gene expression, and roots from control plants were used as a reference sample (set as 1).

**Analysis of Root Peroxidase, Catalase, and Lipoxigenase Activities**

Peroxidase (POD) and catalase (CAT) were extracted and assayed according to Li et al. (2010). Lipoxigenase (LOX) was assayed by the formation of conjugated dienes from linoleic acid according to Axelrod et al. (1981).

**Statistical Analysis**

Differences among four treatment combinations were analyzed by two (B levels) × two (species) ANOVA. Means were separated by the Duncan’s new multiple range test at *P* < 0.05. Significant tests for two means (control and B-excess) were carried out by unpaired *t*-test at *P* < 0.05 level.

**RESULTS**

**Effects of B-Excess on Root B, P, and Total Soluble Protein Concentrations**

Root B concentration was higher in excess B-treated *C. grandis* and *C. sinensis* roots than in controls. Root B level did not differ significantly between the two citrus species at each given B treatment (Figure 1A).

Root P concentration did not significantly differ among four treatment combinations except for a drop in excess B-treated *C. grandis* roots (Figure 1B).

Boron excess only lowered total soluble protein concentration in *C. grandis* roots. Total soluble protein concentration was higher in excess B-treated *C. sinensis* roots than in excess B-treated *C. grandis* but was similar between control roots of the two citrus species (Figure 1C).

**Root B-Excess-Responsive Genes Revealed by cDNA-AFLP**

A total of 256 selective primer combinations were employed to isolate B-excess-responsive genes from B-tolerant *C. sinensis* and B-intolerant *C. grandis* roots. A representative silver-stained cDNA-AFLP gel displaying the differentially expressed TDFs in excess B-treated *C. grandis* and *C. sinensis* roots was presented in Figure 2. As shown in Table 1, we obtained 5873 TDFs from control and B-toxic *C. sinensis* and *C. grandis* roots, 893 (589) of which were presented only in *C. grandis* (*C. sinensis*) roots and 4391 were shared by the both.

A 1.5-fold cut-off was employed to determine up- and down-regulated TDFs, in addition to a *P*-value of <0.05. According to the two criteria, we identified 294 B-excess-responsive TDFs from *C. grandis* and *C. sinensis* roots. After sequencing all these TDFs, we obtained readable sequences from 241 TDFs, 157 of which shared identity with genes encoding known, putative, uncharacterized, predicted, hypothetical, or unnamed proteins, and 84 showed no significant matches (Table 1). The 157 matched TDFs were related to carbohydrate and energy metabolism, cell wall and cytoskeleton metabolism, lipid metabolism, nucleic acid metabolism, protein and amino acid metabolism, stress response, signal transduction, transport, and...
Despite this, great differences existed in B-excess-induced alterations of gene profiles between roots and leaves. We isolated more B-excess-responsive TDFs from C. grandis roots than from C. sinensis, demonstrating that B-excess affected gene expression more in B-intolerant C. grandis roots than in B-tolerant C. sinensis roots (Tables 1, 2). This agrees with our data that B-excess only lowered P and total soluble protein concentrations in C. grandis roots (Figures 1B, C) and also agrees with our report that more B-excess-responsive TDFs were obtained from C. grandis leaves than from C. sinensis leaves (Guo et al., 2014). Despite this, great differences existed in B-excess-induced alterations of gene profiles between roots and leaves. For example, most of B-excess-responsive TDFs were identified only from roots or leaves, and only three TDFs with the same genebank ID (i.e., BAD94010.1, NP_564576.1, and XP_003614394.1) were isolated from roots and leaves of C. sinensis and C. grandis. Moreover, the responses of the three TDFs to B-excess differed between leaves and roots (Table 2; Guo et al., 2014).

**DISCUSSION**

We isolated more B-excess-responsive TDFs from C. grandis roots than from C. sinensis, demonstrating that B-excess affected gene expression more in B-intolerant C. grandis roots than in B-tolerant C. sinensis roots (Tables 1, 2). This agrees with our data that B-excess only lowered P and total soluble protein concentrations in C. grandis roots (Figures 1B, C) and also agrees with our report that more B-excess-responsive TDFs were obtained from C. grandis leaves than from C. sinensis leaves (Guo et al., 2014). Despite this, great differences existed in B-excess-induced alterations of gene profiles between roots and leaves. For example, most of B-excess-responsive TDFs were identified only from roots or leaves, and only three TDFs with the same genebank ID (i.e., BAD94010.1, NP_564576.1, and XP_003614394.1) were isolated from roots and leaves of C. sinensis and C. grandis. Moreover, the responses of the three TDFs to B-excess differed between leaves and roots (Table 2; Guo et al., 2014).

**Effects of B-Excess on Root Carbohydrate and Energy Metabolism**

As shown in Table 2, the mRNA levels of three TDFs encoding cytochrome c oxidase subunit I (TDF #231_1), NADH dehydrogenase subunit 4 (TDF #101_3), and electron transfer flavoprotein-ubiquinone oxidoreductase (TDF #41_2), the components of the mitochondrial respiratory chain, and of two TDFs encoding pyruvate dehydrogenase E1 α subunit (TDF #98_2) and PK (TDF #53_4) involved in glycolysis were lowered in excess B-treated C. sinensis roots, implying that respiration was impaired in these roots. Similarly, both PK (TDF #53_4) and phosphoglycerate kinase (PGK, TDF #185_1) related to glycolysis were inhibited in excess B-treated C. grandis roots. Interestingly, the expression of alternative oxidase (AOX, a cyanide-insensitive terminal oxidase) gene (TDF #150_1) was also down-regulated in excess B-treated C. grandis roots. Unlike the cytochrome pathway, the AOX pathway, which is cyanide-insensitive and can operate when the cytochrome pathway is restricted, plays a role in inhibiting ROS production in plant mitochondria (Popov et al., 1997; Purvis, 1997; Maxwell et al., 1999; Zidenga et al., 2012). Cytochrome P450s (CytP450s), a superfamily of monooxygenases, have a key role in (a)biotic stresses. We found that CytP450 (TDF #137_2) was repressed in excess B-treated C. grandis roots, as obtained on B-toxic Arabidopsis roots (Aquea et al., 2012). Transgenic potato and tobacco plants expressing CytP450 displayed increased monooxygenase activity and enhanced tolerance to oxidative stress after herbicide treatment (Gorinova et al., 2005). Therefore, B-excess-induced down-regulation of AOX and CytP450 in C. grandis roots might contribute to the less B-tolerance of C. grandis.
Effects of B-Excess on Root Cell Wall and Cytoskeleton Metabolism

Cell wall and cytoskeleton metabolism were altered in excess B-treated *C. sinensis* and *C. grandis* roots, as indicated by B-excess-induced alterations of 10 related genes (i.e., 65_1, 151_2, 37_2, 99_2, 50_1, 26_1, 231_3, 138_1, 249_1, and 253_1; Table 2). Similar results have been obtained on excess B-treated citrus leaves (Guo et al., 2014; Sang et al., 2015). This agrees with the reports that B-toxicity affected citrus root cell walls (Huang et al., 2014) and that B-toxicity disturbed actin filament organization, thus impairing the directional transport of cell wall materials and cell wall construction in *Malus domestica* pollen tubes (Fang et al., 2016). *Root Hair Defective 3 (RHD3)*, a gene encoding a putative GTP-binding protein, is required for actin organization, cell wall biosynthesis, and normal cell expansion in *Arabidopsis* roots (Wang et al., 2002; Hu et al., 2003). Overexpression of *PeRHD* in poplar led to less formation of adventitious roots, better-developed lateral roots, and more and longer root hairs (Xu et al., 2012). B-excess-induced up-regulation of *RHD3* in *C. sinensis* roots might be an adaptive response by maintaining root normal growth and development. By contrast, *villin 4* (TDF #50_1), an actin filament bundling protein gene, was down-regulated in excess B-treated *C. grandis* roots (Table 2). Zhang et al. (2011) demonstrated that *Arabidopsis villin 4* was required for normal root hair growth through regulating the organization of actin bundles. The down-regulation of *villin 4* implied that B-excess impaired actin organization in *C. grandis* roots, thus affecting the root growth. The differences in Al-induced alterations of root cell wall and cytoskeleton metabolism between the two citrus species might be responsible for the greater decrease in root dry weight.
TABLE 2 | Homologies of differentially expressed cDNA-AFLP fragments with known gene sequences in database using BLASTN algorithm along their expression patterns in excess B-treated C. grandis (CG) and Citrus sinensis (CS) roots.

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|------------------------|-------------|----------------|
|       |           |            |                 |         |                |                        |             |                |
|       |           | CARBOHYDRATE AND ENERGY METABOLISM |                 |         |                |                        |             |                |
| 232_1 | 286       | Cytochrome c oxidase subunit Vb | Dimocarpus longan | 7.00E-37 | 92              | 46                     | AEQ61828.1  | 0              |
| 101_5 | 179       | NADH dehydrogenase subunit 4, partial (mitochondrion) | Actinidia polygama | 1.00E-26 | 96              | 9                      | CAD60445.1  | 0              |
| 41_2  | 315       | Electron transfer flavoprotein-ubiquinone oxidoreductase | Medicago truncatula | 2.00E-51 | 80              | 17                     | XP_003597528.1 | 0.41           |
| 98_2  | 254       | Pyruvate dehydrogenase E1 alpha subunit | Arabidopsis thaliana | 1.00E-26 | 83              | 16                     | NP_171617.1 | 0.32           |
| 53_4  | 251       | Pyruvate kinase | Arabidopsis thaliana | 5.00E-39 | 96              | 12                     | NP_001078275.1 | 0 0.35         |
| 185_1 | 241       | Phosphoglycerate kinase | Arabidopsis thaliana | 3.00E-15 | 51              | 14                     | NP_178073.1 | 0              |
| 150_1 | 391       | Alternative oxidase | Mangifera indica | 7.00E-33 | 87              | 21                     | CAAB5892.1  | 0              |
| 137_2 | 204       | Cytochrome P450 | Arabidopsis thaliana | 2.00E-19 | 66              | 11                     | AAB36754.1  | 0.29           |
| 9_2   | 201       | Ferredoxin-NADP reductase, root isozyme 2 | Arabidopsis thaliana | 8.00E-30 | 86              | 15                     | NP_173942.1 | 2.25           |
| 26_2  | 314       | Diphospho-CoA kinase | Medicago truncatula | 5.00E-44 | 73              | 26                     | XP_003625901.1 | 2.83           |
| 248_1 | 261       | Sucrose synthase | Citrus unshiu | 2.00E-44 | 100             | 7                      | BAA98049.1  | 0.27           |
| 116_2 | 353       | NADP-dependent D-sorbitol-6-phosphate dehydrogenase | Medicago truncatula | 2.00E-39 | 86              | 27                     | XP_003607080.1 | 2.43           |
|       |           |               |                 |         |                |                        |             |                |
|       |           | CELL WALL AND CYTOSKELETON METABOLISM |                 |         |                |                        |             |                |
| 65_2  | 305       | Root hair defective 3 GTP-binding protein (RHD3) | Arabidopsis thaliana | 4.00E-25 | 84              | 13                     | NP_177439.2 | +              |
| 151_2 | 303       | Alpha-1,4-glucan-protein synthase [UDP-forming], putative | Ricinus communis | 3.00E-60 | 92              | 30                     | XP_002525910.1 | 0.34          |
| 37_2  | 240       | Fucosyltransferase 8 | Arabidopsis thaliana | 2.00E-21 | 63              | 15                     | NP_001077531.1 | 0              |
| 99_2  | 254       | Polygalacturonase-like protein | Arabidopsis thaliana | 3.00E-11 | 54              | 17                     | BAA97779.1  | 0              |
| 50_1  | 255       | Villin 4 | Arabidopsis thaliana | 2.00E-17 | 71              | 6                      | NP_194745.1 | 0              |
| 26_1  | 385       | O-methyltransferase | Citrus sinensis x Citrus reticulata | 1.00E-41 | 65              | 36                     | AP694018.1 | 0              |
| 231_3 | 219       | Beta-D-xylolysidase 4 | Arabidopsis thaliana | 7.00E-29 | 87              | 9                      | NP_201262.1 | 0.46           |
| 138_1 | 415       | Cell wall-associated hydrolase, partial | Medicago truncatula | 5.00E-44 | 96              | 19                     | XP_003637074.1 | +             |
| 249_1 | 384       | Protein SPIRAL1-like2 | Arabidopsis thaliana | 3.00E-27 | 80              | 62                     | NP_177083.1 | 3.72           |
| 253_1 | 346       | S-acetyltransferase | Arabidopsis lyrata subsp.lyrata | 5.00E-39  | 76              | 15                     | NP_002887235.1 | 0.51          |
|       |           | LIPID METABOLISM |                 |         |                |                        |             |                |
| 183_2 | 380       | Glycerol-3-phosphate acyltransferase 9 | Arabidopsis thaliana | 9.00E-62 | 82              | 28                     | NP_568925.1 | 0.29           |
| 44_1  | 95        | Dihydroxyacetone kinase | Arabidopsis thaliana | 4.00E-05 | 84              | 4                      | NP_188404.1 | +              |
| 249_2 | 330       | Coproporphyrinogen III oxidase | Arabidopsis thaliana | 3.00E-55 | 88              | 23                     | NP_171847.4 | 0.33           |
| 24_2  | 328       | Cinnamate 4-hydroxylase | Citrus x paradisi | 7.00E-27 | 100             | 19                     | AAK57011.1 | 0.41           |
| 53_3  | 284       | Lipoygenase | Vitis vinifera | 9.00E-41 | 80              | 10                     | AC217393.1 | 0              |
| 129_3 | 290       | Fatty acid/sphingolipid desaturase | Arabidopsis thaliana | 3.00E-40 | 80              | 17                     | NP_182144.1 | 0 0.05         |
| 150_2 | 379       | Acyl carrier protein | Camellia oleifera | 2.00E-16 | 87              | 53                     | AFK31314.1 | 0              |
| 89_2  | 295       | Acyl carrier protein 3 | Helianthus annuus | 1.00E-30 | 81              | 70                     | ADV16367.1 | 0              |
| 9_1   | 213       | Citrus dioxygenase | Citrus limetta | 6.00E-36 | 94              | 20                     | AER36089.1 | +              |
| 42_1  | 261       | 1,4-dihydroxy-2-naphthoyl-CoA thioesterase 1 | Arabidopsis thaliana | 2.00E-31 | 81              | 32                     | NP_175266.1 | 3.61           |

(Continued)
| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|------------------------|-------------|----------------|
| 103_6 | 152       | JAZ3       | Vitis rupestris  | 2.00E-11| 70             | 13                     | AEP60134.1  | 0.24           |
| 140_2 | 249       | Histone deacetylase 19 | Arabidopsis thaliana | 6.00E-30| 81             | 13                     | NP_001078511.1 | 2.7 |
| 129_1 | 299       | Histone H4  | Arabidopsis thaliana | 1.00E-32| 100            | 45                     | NP_001077477.1 | 2.2 |
| 148_1 | 208       | Zinc finger protein constans-like 5-like | Glycine max | 5.00E-25| 87             | 18                     | NP_001239972.1 | + |
| 82_1  | 213       | DNA binding protein | Arabidopsis lyrata subsp. Lyrata | 2.00E-04| 74             | 5                      | XP_002889547.1 | 0.5 |
| 28_1  | 308       | PREDICTED: ATP-dependent DNA helicase recG-like | Vitis vinifera | 6.00E-15| 53             | 10                     | XP_002280664.2 | 0 |
| 131_4 | 296       | Transcription initiation factor IIb-2 | Arabidopsis thaliana | 2.00E-51| 93             | 23                     | NP_187644.1  | 0.36           |
| 60_1  | 395       | Poly(A)-binding protein | Daucus carota | 5.00E-24| 80             | 16                     | AAK30205.1  | 0 |
| 246_1 | 309       | Transcription factor TCP15 | Arabidopsis thaliana | 1.00E-41| 89             | 19                     | NP_564973.1  | 0 |
| 79_1  | 272       | KNAT4 homeobox protein | Arabidopsis thaliana | 4.00E-47| 99             | 18                     | CAA63131.1  | + |
| 123_2 | 287       | DEAD-box ATP-dependent RNA helicase 20 | Arabidopsis thaliana | 5.00E-30| 77             | 16                     | NP_175911.1  | 4.1 |
| 195_1 | 268       | Ribonucleotide reductase RNR1 like protein, partial | Arabidopsis thaliana | 1.00E-46| 94             | 27                     | CAA69026.1  | 0 |
| 133_4 | 273       | Putative phosphoribosylaminoimidazole carboxylase/AFR carboxylase | Arabidopsis thaliana | 1.00E-40| 80             | 11                     | NP_181305.2  | 0 |
| 134_1 | 308       | Polyphosphatide tract-binding protein 1 | Arabidopsis thaliana | 9.00E-53| 90             | 20                     | NP_186764.1  | 0 |
| 142_1 | 231       | Transcription factor/ transcription initiation factor | Medicago truncatula | 7.00E-24| 74             | 13                     | XP_003597404.1 | 0.53 |
| 252_1 | 310       | AT1G56110, partial | Arabidopsis thaliana | 5.00E-24| 74             | 23                     | BAH20197.1  | 0.33 |
| 81_3  | 325       | Hypothetical protein P0PTR_0001s24865g | Populus trichocarpa | 2.00E-49| 79             | 14                     | XP_002299666.1 | 1.84 |

**Nucleic Acid Metabolism**

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|------------------------|-------------|----------------|
| 85_2  | 356       | Ubiquitin-protein ligase 1 | Arabidopsis thaliana | 4.00E-59| 88%            | 3%                     | AAF36454.1  | 0.38           |
| 132_3 | 384       | E3 ubiquitin-protein ligase MARCH3 | Medicago truncatula | 6.00E-09| 86             | 27                     | XP_003613768.1 | 0 |
| 73_2  | 324       | E3 ubiquitin-protein ligase ATL6 | Arabidopsis thaliana | 8.00E-17| 50             | 23                     | AAD33584.1  | 0 |
| 42_3  | 180       | Ubiquitin, partial | Oryza sativa | 2.00E-26| 96             | 46                     | AAL77200.1  | + |
| 86_1  | 398       | Ubiquitin-protein ligase, putative | Ricinus communis | 8.00E-25| 57             | 18                     | XP_002520193.1 | 0.43 |
| 112_1 | 461       | E3 ubiquitin-protein ligase UFL5 | Medicago truncatula | 3.00E-42| 70             | 18                     | XP_003594229.1 | 0 |
| 27_1  | 333       | ARM repeat superfamily protein | Arabidopsis thaliana | 3.00E-52| 83             | 66                     | NP_198149.2  | 0 |
| 230_1 | 258       | Aspartic protease, partial | Dimocarpus longan | 1.00E-05| 71             | 26                     | AEJ76922.1  | + |
| 62_1  | 326       | Aspartic proteinase nepenthesin-1 | Medicago truncatula | 1.00E-37| 77             | 19                     | XP_003627883.1 | 0 |

**Protein and Acid Metabolism**

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|------------------------|-------------|----------------|
| 61_1  | 335       | Protein ASPARTIC PROTEASE IN GUARD CELL 1 | Arabidopsis thaliana | 3.00E-40| 65             | 18                     | NP_188478.1  | 1.79 |
| 118_5 | 208       | Serine carboxypeptidase I-3 | Medicago truncatula | 3.00E-22| 72             | 14                     | XP_003592243.1 | 0.21 |
| 41_1  | 141       | Drought-inducible cysteine proteinase RD19A precursor | Arabidopsis thaliana | 5.00E-16| 86             | 35                     | BAD94010.1  | 0 |
| 24_1  | 352       | Gamma-glutamyl hydrolase 2 | Arabidopsis thaliana | 2.00E-34| 74             | 25                     | NP_585186.2  | 0.35 |
| 253_4 | 264       | Protein disulfide isomerase | Zea mays | 7.00E-23| 64             | 13                     | ACG47473.1  | 0.43 |
| 184_1 | 267       | Protein disulfide isomerase-like 1-1 | Arabidopsis thaliana | 2.00E-09| 56             | 17                     | NP_849668.1  | 0.38 |
| 12_1  | 361       | Elongation factor 1-alpha, partial | Arabidopsis thaliana | 2.00E-21| 93             | 60                     | BAD94755.1  | 2.04 |
| 121_2 | 160       | 40S ribosomal protein S8 | Zea mays | 3.00E-19| 98             | 19                     | NP_001105391.1 | 2.52 |

(Continued)
**TABLE 2** | Continued

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|-----------------------|-------------|---------------|
| 48_1  | 303       | 40S ribosomal protein S4 | Medicago truncatula | 2.00E-52 | 90 | 24 | XP_003604568.1 | 0.44 |
| 246_5 | 228       | 40S ribosomal protein S2 | Arabidopsis lyrata subsp. Lyrata | 5.00E-29 | 87 | 27 | XP_002878157.1 | 0 |
| 9_3   | 198       | 60S ribosomal protein L7a | Zea mays | 2.00E-15 | 91 | 19 | ACG47588.1 | + + |
| 153_2 | 261       | 40S ribosomal protein S5 | Capsicum annuum | 4.00E-44 | 100 | 31 | AAR89617.1 | 0 0.39 |
| 80_2  | 320       | 50S ribosomal protein L2, chloroplastic | Citrus sinensis | 3.00E-63 | 99 | 62 | YP_740517.1 | 0 0 |
| 64_2  | 291       | 60S ribosomal protein L10-1 | Arabidopsis thaliana | 2.00E-54 | 90 | 30 | NP_565945.2 | + |
| 140_1 | 335       | 40S ribosomal protein S29 | Zea mays | 9.00E-26 | 86 | 74 | ACQ30830.1 | 3.12 |
| 38_1  | 290       | 60S ribosomal protein L15-1 | Arabidopsis thaliana | 2.00E-47 | 84 | 33 | NP_193405.1 | 0.41 |
| 14_2  | 225       | Translation initiation factor eIF-4A1 | Arabidopsis thaliana | 1.00E-37 | 100 | 13 | CAC43286.1 | 0 |
| 229_1 | 327       | Methionine synthase 2 | Arabidopsis thaliana | 3.00E-59 | 92 | 12 | NP_187028.1 | 0 0.46 |
| 144_2 | 266       | Threonyl-tRNA synthetase | Medicago truncatula | 2.00E-41 | 88 | 11 | XP_003601575.1 | 0 0.22 |
| 5_1   | 354       | S-adenosyl-L-homocysteine hydrolase | Gossypium hirsutum | 2.00E-27 | 89 | 19 | ACJ11250.1 | 0 0 |
| 229_2 | 192       | Bilinfunctional aminoacyl-tRNA synthetase, putative, expressed | Oryza sativa Japonica Group | 5.00E-30 | 96 | 10 | ABA97740.1 | 0.34 |

**STRESS RESPONSE**

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|-----------------------|-------------|---------------|
| 182_3 | 214       | Homogentisate phytyltransferase 1 | Arabidopsis thaliana | 3.00E-14 | 51 | 12 | NP_849984.1 | + + |
| 64_1  | 309       | Obg-like ATPase 1 | Arabidopsis thaliana | 5.00E-51 | 87 | 20 | NP_174346.1 | 0 |
| 99_3  | 238       | Peroxidase | Medicago truncatula | 3.00E-22 | 60 | 14 | XP_003615990.1 | 0 |
| 231_5 | 188       | Catalase, partial | Citrus maxima | 9.00E-31 | 91 | 19 | ACY30463.1 | 0 0 |
| 30_1  | 320       | DJ-1 family protein | Arabidopsis lyrata subsp.lyrata | 9.00E-28 | 73 | 24 | XP_002894433.1 | 0.24 |
| 233_5 | 141       | Heat shock protein 70 | Nicotiana benthamiana | 3.00E-05 | 75 | 33 | BAD02271.1 | + |
| 237_1 | 305       | BAG family molecular chaperone regulator 7 | Arabidopsis thaliana | 4.00E-14 | 67 | 16 | NP_201045.1 | 0.35 |
| 131_1 | 332       | Putative senescence-associated protein, partial | Trichosanthes dioica | 5.00E-05 | 100 | 100 | ABN50029.1 | + |
| 17_1  | 296       | Putative senescence-associated protein, partial | Ipomoea nil | 6.00E-52 | 93 | 25 | BAF46313.1 | 2.52 |
| 104_3 | 171       | WD repeat phosphoinositide-interacting-like protein | Medicago truncatula | 4.00E-21 | 96 | 11 | XP_005591137.1 | 3.15 |
| 77_5  | 112       | Universal stress protein A-like protein | Medicago truncatula | 2.00E-09 | 74 | 12 | XP_003616191.1 | 0.37 |
| 140_3 | 220       | ACR toxin-sensitivity inducing protein (mitochondrion) | Citrus jambhiri | 4.00E-10 | 96 | 10 | BAB85481.1 | + |
| 251_5 | 266       | Ankyrin-like protein | Arabidopsis thaliana | 7.00E-38 | 82 | 9 | BAB10271.1 | 0 |
| 146_5 | 285       | Leucine-rich repeat containing protein, putative | Ricinus communis | 1.00E-20 | 59 | 8 | XP_002523984.1 | 0 |

**SIGNAL TRANSDUCTION**

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|-----------------------|-------------|---------------|
| 35_1  | 341       | ATP/GTP/Ca++ binding protein | Cucumis melo subsp.melo | 2.00E-44 | 74 | 18 | ABP67417.1 | 3.04 |
| 80_3  | 194       | Calcium-dependent lipid-binding domain-containing protein | Arabidopsis thaliana | 2.00E-19 | 80 | 13 | NP_564576.1 | 0.41 |
| 67_2  | 232       | Calcium-binding EF-hand-containing protein | Arabidopsis thaliana | 2.00E-07 | 52 | 5 | NP_173582.2 | 0.49 |

(Continued)
TABLE 2 | Continued

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|-----------------------|-------------|----------------|
| 35_2 | 267 | Calcium ion binding protein, putative | Ricinus communis | 7.00E-32 | 67 | 18 | XP_002534034.1 | 0.48 |
| 103_2 | 207 | Serine/threonine-protein kinase | Nicotiana attenuata | 3.00E-20 | 71 | 8 | AEI84329.1 | + |
| 251_3 | 301 | Leucine-rich repeat receptor-like protein kinase | Medicago truncatula | 4.00E-34 | 68 | 15 | XP_003600547.1 | 0 0 |
| 53_2 | 350 | ATP binding/kinase/protein serine/threonine kinase | Vitis vinifera | 3.00E-50 | 80 | 12 | CAQ58615.1 | + |
| 122_1 | 343 | Receptor-like protein kinase-like protein | Glycine max | 4.00E-46 | 77 | 13 | ACM89521.1 | 0 |
| 103_3 | 207 | 14-3-3 protein | Litchi chinensis | 6.00E-33 | 95 | 19 | AEE25651.1 | 0 |
| 117_1 | 184 | Auxin-responsive protein | Gossypium hirsutum | 1.00E-07 | 54 | 15 | NP_190378.2 | + + |
| 39_1 | 330 | Phytochrome A, partial | Populus tremula | 2.00E-51 | 86 | 7 | NP_199794.1 | 0 0 |
| TRANSPORT | | | | | | | |
| 251_2 | 316 | H^+^-ATPase 4, plasma membrane-type | Arabidopsis thaliana | 3.00E-23 | 92 | 10 | NP_190378.2 | + + |
| 76_3 | 270 | Exocyst complex component 84B | Arabidopsis thaliana | 8.00E-25 | 64 | 9 | NP_199794.1 | 0 0 |
| 7_7 | 448 | Vacuolar membrane ATPase subunit c^* | Citrus limon | 1.00E-71 | 99 | 50 | AA073433.1 | 0 |
| 151_1 | 336 | Exocyst subunit exo70 family protein B1 | Arabidopsis thaliana | 1.00E-50 | 82 | 15 | NP_200651.1 | 0 |
| 135_1 | 326 | ADP ribosylation factor | Medicago truncatula | 2.00E-50 | 100 | 88 | XP_003591354.1 | 0.49 |
| 246_4 | 228 | Peroxin 7 | Arabidopsis thaliana | 5.00E-07 | 75 | 16 | NP_174220.1 | 0.45 |
| 39_2 | 216 | Protein transport protein SEC61 gamma subunit | Medicago truncatula | 3.00E-04 | 90 | 64 | XP_003617671.1 | 0 |
| 183_3 | 236 | ABC transporter I family member 20 | Arabidopsis thaliana | 1.00E-12 | 69 | 20 | NP_196847.1 | 0.42 |
| 253_3 | 318 | Metal tolerance protein | Carica papaya | 7.00E-52 | 89 | 26 | ADI24923.1 | 0 |
| 19_1 | 316 | Sec61 transport protein | Populus trichocarpa | 6.00E-54 | 88 | 16 | XP_002331716.1 | 0.5 |
| 141_2 | 272 | Synaptobrevin-like protein | Medicago truncula | 6.00E-35 | 83 | 20 | XP_003617238.1 | 0 |
| 79_3 | 196 | PRA1 (prenylated RAB acceptor) family protein | Medicago truncula | 1.00E-18 | 65 | 18 | XP_003591354.1 | 0.31 |
| 10_1 | 147 | Protein tolB | Medicago truncula | 1.00E-09 | 72 | 7 | XP_003637684.1 | 0.37 |
| OTHERS | | | | | | | |
| 98_3 | 331 | Rubber elongation factor protein | Arabidopsis thaliana | 2.00E-23 | 63 | 31 | NP_187201.1 | + |
| 133_5 | 259 | FRIGIDA-like protein | Arabidopsis thaliana | 9.00E-25 | 69 | 12 | NP_850923.1 | 0 |
| 121_1 | 248 | Limonoid UDP-glucosyltransferase | Citrus maxima | 5.00E-11 | 70 | 16 | NP_186847.1 | 0.42 |
| 77_4 | 318 | ATP sulfurylase 1 | Arabidopsis thaliana | 2.00E-54 | 97 | 18 | NP_188929.1 | 0 |
| 38_2 | 134 | FAD-binding berberine family protein | Medicago truncula | 2.00E-13 | 85 | 6 | XP_003594602.1 | 0.41 |
| 189_1 | 309 | Probable methyltransferase PMT14 | Arabidopsis thaliana | 4.00E-37 | 62 | 15 | NP_561447.1 | 0.3 |
| 41_3 | 398 | 1-deoxy-D-xylulose-5-phosphate synthase, partial | Buxa orellana | 2.00E-65 | 87 | 68 | AAO29063.1 | 1.9 |
| 173_1 | 286 | DPP6 N-terminal domain-like protein | Arabidopsis thaliana | 4.00E-21 | 70 | 12 | NP_561447.1 | 0.3 |
| 66_1 | 284 | F22GS.28 | Arabidopsis thaliana | 6.00E-36 | 68 | 12 | AAF79556.1 | + |
| 147_4 | 254 | PREDICTED: uncharacterized protein LOC100264520 | Vitis vinifera | 3.00E-38 | 84 | 24 | XP_002276822.1 | 0.51 |
| 109_3 | 277 | Unnamed protein product, partial | Vitis vinifera | 1.00E-19 | 64 | 50 | CB11118.3 | 0.28 |
| 153_3 | 226 | Predicted protein | Arabidopsis lyrata subsp. | 2.00E-16 | 69 | 46 | XP_002871569.1 | 1.78 | (Continued)
### TABLE 2 | Continued

| TDF # | Size (bp) | Homologies | Organism origin | E-value | Similarity (%) | Sequence coverage (%) | Genebank ID | Ratio of BE/CK |
|-------|-----------|------------|-----------------|---------|----------------|-----------------------|-------------|----------------|
| 22_4  | 287       | Hypothetical protein VITISV_014691 | Vitis vinifera | 2.00E-27 | 65             | 21                    | CAN71367.1 | 2.32           |
| 158_1 | 177       | PREDICTED: uncharacterized protein LOC100255693 | Vitis vinifera | 5.00E-09 | 51             | 13                    | XP_002279857.1 | 1.33         |
| 21_1  | 231       | AT5G10200-like protein | Arabidopsis arenosa | 1.00E-31 | 90             | 9                     | ACG44505.1 | +             |
| 163_3 | 228       | PREDICTED: uncharacterized protein LOC100262433 | Vitis vinifera | 2.00E-30 | 77             | 11                    | XP_002284886.1 | 0            |
| 27_2  | 267       | Predicted protein | Populus trichocarpa | 5.00E-27 | 63             | 30                    | XP_002330640.1 | 0            |
| 116_1 | 401       | Hypothetical protein MTR_1g005920 | Medicago truncatula | 3.00E-25 | 79             | 75                    | XP_003588318.1 | 0.39         |
| 163_2 | 330       | Unnamed protein product | Vitis vinifera | 8.00E-07 | 69             | 6                     | CBI37596.3 | + 0           |
| 170_2 | 212       | Predicted protein | Populus trichocarpa | 7.00E-07 | 68             | 14                    | XP_002309501.1 | 0            |
| 77_6  | 230       | Hypothetical protein MTR_5g051130 | Medicago truncatula | 4.00E-11 | 97             | 6                     | XP_003614394.1 | 0.51         |
| 105_2 | 277       | PREDICTED: UPF0197 transmembrane protein C11orf10 | Glycine max | 3.00E-25 | 87             | 42                    | XP_003549239.1 | 2.13         |
| 108_2 | 144       | Hypothetical protein SORBIDRAFT_0351s002020 | Sorghum bicolor | 7.00E-16 | 100            | 28                    | XP_002489033.1 | + 0           |
| 109_5 | 178       | Unknown | Glycine max | 6.00E-15 | 89             | 57                    | ACU14517.1 | +             |
| 118_3 | 287       | Hypothetical protein MTR_5g051140 | Medicago truncatula | 1.00E-14 | 93             | 8                     | XP_003614395.1 | 2.47         |
| 170_1 | 296       | Unknown | Glycine max | 5.00E-31 | 95             | 30                    | ACU24256.1 | 1.8 2.11      |
| 173_3 | 213       | Hypothetical protein MTR_5g051120 | Medicago truncatula | 1.00E-25 | 88             | 18                    | XP_003614393.1 | 1.73         |
| 178_1 | 306       | Hypothetical protein Lilium longiflorum | 2.00E-25 | 96             | 100                   | ABO20854.1 | + 0           |
| 193_3 | 244       | Hypothetical protein MTR_4g091430 | Medicago truncatula | 3.00E-11 | 57             | 48                    | XP_003608262.1 | +             |
| 228_1 | 306       | Hypothetical protein Arabidopsis thaliana | 1.00E-39 | 100            | 11                    | BAF01964.1 | 1.32 +         |
| 237_3 | 204       | PREDICTED: uncharacterized protein LOC100800109 | Glycine max | 2.00E-31 | 92             | 54                    | XP_003541224.1 | +             |
| 249_5 | 224       | Hypothetical protein | Zea mays | 4.00E-20 | 98             | 24                    | ACG27665.1 | 1.38         |
| 37_3  | 217       | Hypothetical protein SORBIDRAFT_0070s002020 | Sorghum bicolor | 3.00E-03 | 82             | 72                    | XP_002489102.1 | 3.01 0.39    |
| 53_5  | 165       | Hypothetical protein SORBIDRAFT_1994s002010 | Sorghum bicolor | 2.00E-10 | 100            | 28                    | XP_002489133.1 | 2.73         |
| 6_3   | 218       | Hypothetical protein MTR_5g051030 | Medicago truncatula | 8.00E-25 | 95             | 9                     | XP_003614386.1 | 2.06         |
| 85_1  | 369       | Hypothetical protein SORBIDRAFT_1368s002010 | Sorghum bicolor | 7.00E-52 | 91             | 66                    | XP_002489471.1 | 0.47         |
| 94_1  | 222       | Hypothetical protein MTR_8g040260 | Medicago truncatula | 2.00E-06 | 51             | 31                    | XP_003627937.1 | +             |
| 146_4 | 245       | Putative uncharacterized protein Sb00351s002020 | Sorghum bicolor | 3.00E-12 | 97             | 47                    | XP_002489033.1 | 0.54         |
| 252_4 | 190       | Conserved hypothetical protein Ricinus communis | 2.00E-09 | 83             | 19                    | XP_002532022.1 | 0.38         |
| 133_7 | 217       | Hypothetical protein SORBIDRAFT_0227s002010 | Sorghum bicolor | 2.00E-15 | 97             | 48                    | XP_002489051.1 | + 0.41       |

Data are means of three replicates; Expression ratio, + indicates that TDFs were only isolated from excess B-treated roots. A, Number; BE, B-excess; CK, Control. Functional classification was performed based on the information reported for each sequence by The Gene Ontology (http://amigo1.geneontology.org/cgi-bin/amigo/go.cgi) and Uniprot (http://www.uniprot.org/). Ratio of BE/CK was obtained by gel image analysis performed with PDQuest version 8.0.1 (Bio-Rad, Hercules, CA, USA). B-excess-responsive TDFs shared by the two citrus species were highlighted in bold.
in B-toxic *C. grandis* seedlings relative to B-toxic *C. sinensis* ones (Guo et al., 2014).

### Effects of B-Excess on Root Lipid Metabolism

We found that all these differentially expressed TDFs (i.e., TDFs #183_2, 249_2, 24_2, 53_3, 129_3, 150_2, 89_2, and 103_6) related to lipid metabolism were inhibited in excess B-treated *C. grandis* and *C. sinensis* roots except for three TDFs (i.e., TDFs #44_1, 9_1, and 42_1; Table 2), implying that lipid metabolism pathway in these roots was upset. Glycerol-3-phosphate acyltransferase (GPAT), the first enzyme for the biosynthesis of glycerolipids in all organisms, plays crucial roles in basic cellular metabolism. *AtGPAT9* is required for the biosynthesis of membrane lipids and oils. A knockout mutant of *AtGPAT9* was homozygous lethal (Shockey et al., 2016). We found that *GPAT9* (TDF #183_2) was down-regulated in excess B-treated *C. grandis* roots (Table 2), suggesting that B-toxicity impaired membrane lipid biosynthesis, thus lowering B-tolerance of *C. grandis*.

Lipoxygenase (LOX) reactions may initiate the generation of ROS, thus leading to the loss of membranes (Pérez-Gilabert et al., 2001). Inal et al. (2009) found that B-excess increased malondialdehyde (MDA) level and LOX activity, and silicon (Si) enhanced B-tolerance by decreasing B-excess-induced increases in MDA level and LOX activity in barely. B-excess downregulated (did not significantly affect) LOX expression in *C. grandis* (*C. sinensis*) roots (Table 2) but increased (did not significantly affect) LOX activity in *C. grandis* (*C. sinensis*) roots (Figure 5C). Therefore, the observed higher LOX activity might lower the B-tolerance of *C. grandis*.

We found that *citrus dioxygenase* (TDF #9_1) was up-regulated in excess B-treated *C. sinensis* roots (Table 2). Alpha-dioxygenase (α-DOX) functions in protecting tissues against oxidative damage and cell death (Tirajoh et al., 2005). Therefore, *citrus dioxygenase* might be involved in the B-tolerance of *C. sinensis*.

### Effects of B-Excess on Root Nucleic Acid Metabolism

Boron-toxicity affects nucleic acid metabolic process in higher plants (Kasajima and Fujiwara, 2007; Sakamoto et al., 2011; Guo et al., 2014). As expected, three up- (i.e., TDFs #140_2, 129_1 and 148_1) and five down-regulated (i.e., TDFs #82_1, 28_1, 131_4, 60_1 and 246_1) TDFs, and three up- (i.e., TDFs #79_1, 123_2 and 81_3) and six down-regulated (TDFs #246_1, 195_1, 133_4, 134_1, 142_1, and 252_1) TDFs related to nucleic acid metabolism were isolated from excess B-treated *C. sinensis* and *C. grandis* roots, respectively (Table 2). Histone deacetylases and histone acetyltransferases, which catalyze histone acetylation and deacetylation, respectively, play a key role in the regulation of gene and genome activity. *Arabidopsis* histone deacetylase 19 (AtHDA19 or AtHD1) plays a role in plant growth and development. Reduction in *AtHDA19* expression by T-DNA insertion or antisense inhibition in *Arabidopsis* caused various developmental abnormalities such as suppression of apical dominance, early senescence, and flower defects (Tian and Chen, 2001; Tian et al., 2003). Recently, Chen et al. (2015) obtained a total of 86 P-deficiency-responsive genes, which showed lower expression in AtHDA19-RNAi *Arabidopsis* plants relative to 35S:AtHDA19 lines. Kaya et al. (2009) observed that high-B-treated tomato leaves had lower P concentration, and that supplementary P alleviated high-B-induced decreases in plant growth and fruit yield. B-toxicity decreased P uptake in wheat (Singh et al., 1990). Therefore, excess B-induced up-regulated of HDA19 (TDF #140_2) in *C. sinensis* roots (Table 2) might be an adaptive response by the maintenances of P homeostasis. This agrees with our report that B-toxicity only reduced P level for the biosynthesis of glycerolipids in all organisms, plays crucial roles in basic cellular metabolism. *AtGPAT9* was required for the biosynthesis of membrane lipids and oils. A knockout mutant of *AtGPAT9* was homozygous lethal (Shockey et al., 2016). We found that *GPAT9* (TDF #183_2) was down-regulated in excess B-treated *C. grandis* roots (Table 2), suggesting that B-toxicity impaired membrane lipid biosynthesis, thus lowering B-tolerance of *C. grandis*.

Lipoxygenase (LOX) reactions may initiate the generation of ROS, thus leading to the loss of membranes (Pérez-Gilabert et al., 2001). Inal et al. (2009) found that B-excess increased malondialdehyde (MDA) level and LOX activity, and silicon (Si) enhanced B-tolerance by decreasing B-excess-induced increases in MDA level and LOX activity in barely. B-excess downregulated (did not significantly affect) LOX expression in *C. grandis* (*C. sinensis*) roots (Table 2) but increased (did not significantly affect) LOX activity in *C. grandis* (*C. sinensis*) roots (Figure 5C). Therefore, the observed higher LOX activity might lower the B-tolerance of *C. grandis*.

We found that *citrus dioxygenase* (TDF #9_1) was up-regulated in excess B-treated *C. sinensis* roots (Table 2). Alpha-dioxygenase (α-DOX) functions in protecting tissues against oxidative damage and cell death (Tirajoh et al., 2005). Therefore, *citrus dioxygenase* might be involved in the B-tolerance of *C. sinensis*.
in C. grandis leaves (Guo et al., 2014) and roots (Figure 1B). Similarly, histone H4 (TDF #129_1) was up-regulated in excess B-treated C. sinensis roots (Table 2), which agrees with the reports that histone H4 was induced in Al-toxic rice roots (Mao et al., 2004) and Al-toxicity increased B level and decreased P level in plant roots, stems, and leaves (Jiang et al., 2009a,b). Histones H2A, H2B, H3, and H4, the core component of nucleosome, play crucial roles in chromosomal stability, gene regulation, and DNA repair and replication (Turner, 2002; Drury et al., 2012; Zhu et al., 2012). Sakamoto et al. (2011) demonstrated that plant condensin II played a crucial role in the tolerance of Arabidopsis to B-toxicity by alleviating DNA damage. Therefore, the up-regulation of histone H4 in excess B-treated C. sinensis roots (Table 2) might contribute to the B-tolerance of C. sinensis by preventing genome from damage resulting from B-excess.

Ribonucleotide reductase (RNR), an enzyme comprising two R1 and two R2, plays a key role in DNA repair and replication (Chabouté et al., 1998; Wang and Liu, 2006). The down of RNR1 like protein (TDF #195_1) in excess B-treated C. grandis roots (Table 2) implied that B-toxicity impaired DNA repair and replication, thus lowering the B-tolerance of C. grandis. Similarly, the expression levels of phosphoribosylaminomimidazole carboxylase/AIR carboxylase (TDF #133_4) involved in nucleotide biosynthesis were down-regulated in excess B-treated C. grandis roots.

**Effects of B-Excess on Root Protein and Amino Acid Metabolism**

As shown in Table 2, we isolated 10 down-regulated (i.e., TDFs #85_2, 132_3, 73_2, 86_1, 112_1, 27_1, 62_1, 118_5, 41_1, and 24_1) and three up-regulated (i.e., TDFs #42_3, 230_1, and 61_1) TDFs involved in protein degradation, two down-regulated TDFs (i.e., TDFs #253_4 and 184_1,) involved in protein folding and stability, five up-regulated (i.e., TDFs #12_1, 121_2, 9_3, 64_2, and 140_1) and six down-regulated (i.e., TDFs #48_1, 246_5, 153_2, 80_2, 38_1, and 14_2) TDFs involved in protein biosynthesis, and four down-regulated TDFs (i.e., TDFs #229_1, 144_2, 5_1, and 229_2) involved in amino acid metabolism. Obviously, protein and amino acid metabolism was impaired in B-toxic citrus roots.

**Effects of B-Excess on Root Stress-Related Gene Expression**

Boron-excess causes the accumulation of ROS, thus resulting in oxidative and membrane damage in plant roots and leaves (Cervilla et al., 2007; Pandey and Archana, 2013; Martinez-Cuenca et al., 2015). An antioxidant response has been suggested to abate B-toxic damage in some higher plants (Gunes et al., 2006). Here, we isolated one up-regulated homogentisate phytyltransferase 1 (HPT1; TDF #182_3) from excess B-treated C. grandis and C. sinensis roots (Table 2). HPT catalyzes the
committed step in the biosynthesis of tocopherol (vitamin E). Collakova and DellaPenna (2003) showed that transgenic Arabidopsis plants overexpressing HPT1 had an elevated total tocopherol level in seed and leaves. The up-regulation of HPT1 agrees with the increased requirement for the detoxification of ROS in excess B-treated C. grandis and C. sinensis roots. In addition, Obg-like ATPase 1 (YchF1; TDF #64_1), a negative regulator of the antioxidant response (Cheung et al., 2013), was down-regulated in excess B-treated C. grandis roots. However, POD (TDF #99_3) in C. grandis roots and CAT (TDF #231_5) in C. grandis and C. sinensis roots were down-regulated by B-excess. Enzyme activity analysis showed that B-excess lowered CAT and POD activities in C. grandis roots but did not significantly affect their activities in C. sinensis roots (Figures 5A,B). Besides, the expression level of an antioxidant protein gene (Xu and Möller, 2010; Xu et al., 2010), DJ-1 family protein (TDF #30_1) was reduced in excess B-treated C. grandis roots (Table 2). Obviously, excess B-treated C. sinensis roots kept higher antioxidant capacity than excess B-treated C. grandis roots.

Two putative senescence-associated protein genes (i.e., TDFs #131_1 and 17_1) were induced by B-excess in C. grandis roots (Table 2), suggesting that senescence was accelerated in these roots. This agrees with our report that B-excess affected root growth more in C. grandis than in C. sinensis seedlings (Guo et al., 2014). Similarly, autophagic cell death might be accelerated in excess B-treated C. grandis roots, as indicated by the enhanced expression level of WD repeat phosphoinositide-interacting-like protein (WILP; TDF #104_3) in these roots (Table 2). This agrees with our report that B-excess led to cell death in C. grandis roots (Huang et al., 2014).

**Effects of B-Excess on Root Signal Transduction**

Calcium (Ca) signaling plays vital roles in plant responses to various stresses (Knight, 2000). Ca can alleviate B-toxicity and high-B inhibits Ca uptake in higher plants (Singh et al., 1990; Túran et al., 2009; Siddiqui et al., 2013). Hence, genes related to Ca$^{2+}$ signal transduction should be altered in excess B-treated citrus roots. As expected, we isolated one up-regulated TDF: ATP/GTP/Ca$^{2+}$ binding protein [also called as mitochondrial Rho GTPase (MIRO)] from B-toxic C. sinensis roots (Table 2). Jayasekaran et al. (2006) found that the transcript level of MIRO2 was elevated in ABA- and salt-treated Arabidopsis plants, and that the knock-out mutants of MIRO2 were less tolerance to ABA, salt, and osmotic treatments than the wild-type. By contrast, we only isolated three down-regulated TDFs (i.e., TDFs #80_3, 67-2, and 35_2) involved in Ca signals from B-toxic C. grandis roots. Therefore, Ca$^{2+}$-mediated signal played a role in the tolerance of citrus plants to B-toxicity.

**Effects of B-Excess on Transport Processes in Root Cells**

As shown in Table 2, the mRNA levels of all the 13 differentially expressed TDFs related to cellular transport were reduced in excess B-treated C. grandis and C. sinensis roots except for one up-regulated H+ -ATPase 4 (AHA4, TDF #251_2), indicating that the transport of some substances (i.e., ion and proteins) was altered in these roots. This disagrees with our report that most of the differentially expressed genes involved in cellular transport were up-regulated in excess B-treated C. grandis leaves and three up- and three down-regulated genes were isolated from excess B-treated C. sinensis leaves (Guo et al., 2014).

ABC transporter I family member 20 (ABC120; TDF #183_2) was down-regulated in excess B-treated C. grandis roots (Table 2). This agrees with our report that ABC1 family protein (AT5g24810) was repressed in high-B-treated C. grandis leaves (Guo et al., 2014). In eukaryotic organisms, ABC transporters have been shown to play a role in the efflux (extracellular or intracellular into the vacuoles) of potentially toxic compounds (i.e., heavy metals, alkaloids, and organic anions; Gaedeke et al., 2001). Transgenic Arabidopsis plants overexpressing AtPDR8 encoding an ABC transporter displayed higher Cd- and Pb-tolerance accompanied by less accumulation of Cd in the shoots or roots. By contrast, mutants were less tolerance to both the metals accompanied by more accumulation of Cd (Kim et al., 2007). Therefore, the down-regulation of ABCI20 and ABCI family protein in roots and leaves might reduce the efflux of B from the PM, thus increasing free B level and lowering C. grandis B-tolerance. Interestingly, middle leaves from excess B-treated C.
**CONCLUSIONS**

We used cDNA-AFLP to investigate comparatively the effects of B-excess on gene profiles in B-tolerant *C. sinensis* and B-intolerant *C. grandis* roots, and isolated 20 up- and 52 down-regulated, and 44 up- and 66 down-regulated TDFs from excess B-treated *C. sinensis* and *C. grandis* roots, respectively, thereby demonstrating that gene expression was less affected in the former than in the latter. Besides, B-excess only lowered P and total soluble protein concentrations in *C. grandis* roots. Obviously, *C. sinensis* seedlings had higher B-tolerance than *C. grandis* ones. Our results suggested that the following several aspects led to the difference in the B-tolerance between the both, including: (a) AOX and CytoP450 were down-regulated only in excess B-treated *C. grandis* roots; (b) B-excess up-regulated RHD3 in *C. sinensis* roots and down-regulated villin4 in *C. grandis* roots, and accordingly, root dry weight was less reduced by B-excess in the former; (c) antioxidant systems were impaired in excess B-treated *C. grandis* roots, hence accelerating root senescence; (d) TDFs involved in Ca^2+ signals were down-regulated in excess B-treated *C. grandis* roots and up-regulated in excess B-treated *C. sinensis* roots. In addition, B-excess-responsive genes related to lipid (i.e., GPAT9 and citrus dioxygenase) and nucleic acid (i.e., histone deacetylase 19, histone 4, and RNR1 like protein) metabolisms also possibly contributed to the difference in the B-tolerance between the both. Our data provided some new clues for our understanding of the molecular mechanisms on citrus B-toxicity and B-tolerance.

**AUTHOR CONTRIBUTIONS**

PG carried out most of the experiments and drafted the manuscript. YQ participated in the design of the study. LY participated in the design of the study and coordination. XY participated in the measurement of B and P. JH participated in the statistical analysis. LC designed and directed the study and revised the manuscript. All authors have read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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