cDNA-AFLP analysis reveals the adaptive responses of citrus to long-term boron-toxicity

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Abstract

Background: Boron (B)-toxicity is an important disorder in agricultural regions across the world. Seedlings of ‘Sour pummelo’ (Citrus grandis) and ‘Xuegan’ (Citrus sinensis) were fertigated every other day until drip with 10 μM (control) or 400 μM (B-toxic) H3BO3 in a complete nutrient solution for 15 weeks. The aims of this study were to elucidate the adaptive mechanisms of citrus plants to B-toxicity and to identify B-tolerant genes.

Results: B-toxicity-induced changes in seedlings growth, leaf CO2 assimilation, pigments, total soluble protein, malondialdehyde (MDA) and phosphorus were less pronounced in C. sinensis than in C. grandis. B concentration was higher in B-toxic C. sinensis leaves than in B-toxic C. grandis ones. Here we successfully used cDNA-AFLP to isolate 67 up-regulated and 65 down-regulated transcript-derived fragments (TDFs) from B-toxic C. grandis leaves, whilst only 31 up-regulated and 37 down-regulated TDFs from B-toxic C. sinensis ones, demonstrating that gene expression is less affected in B-toxic C. sinensis leaves than in B-toxic C. grandis ones. These differentially expressed TDFs were related to signal transduction, carbohydrate and energy metabolism, nucleic acid metabolism, protein and amino acid metabolism, lipid metabolism, cell wall and cytoskeleton modification, stress responses and cell transport. The higher B-tolerance of C. sinensis might be related to the findings that B-toxic C. sinensis leaves had higher expression levels of genes involved in photosynthesis, which might contribute to the higher photosynthesis and light utilization and less excess light energy, and in reactive oxygen species (ROS) scavenging compared to B-toxic C. grandis leaves, thus preventing them from photo-oxidative damage. In addition, B-toxicity-induced alteration in the expression levels of genes encoding inorganic pyrophosphatase 1, AT4G01850 and methionine synthase differed between the two species, which might play a role in the B-tolerance of C. sinensis.

Conclusions: C. sinensis leaves could tolerate higher level of B than C. grandis ones, thus improving the B-tolerance of C. sinensis plants. Our findings reveal some novel mechanisms on the tolerance of plants to B-toxicity at the gene expression level.

Keywords: Boron-tolerance, Boron-toxicity, cDNA-AFLP, Citrus grandis, Citrus sinensis, Photosynthesis

Background

Although boron (B) is a micronutrient element required for normal growth and development of higher plants, it is harmful to plants when present in excess. Whilst of lesser importance than B-deficiency (a widespread problem in many agricultural crops), B-toxicity is also an important problem in agricultural regions across the world, which citrus trees are cultivated [1-3]. Despite the importance of B-toxicity for crop productivity, the mechanisms by which plants respond to B-toxicity are poorly understood yet. Recently, increasing attention has been paid to plant B-toxicity as a result of the increased demand for desalinated water, in which the B level may be too high for healthy irrigation of crops [4].

Alteration of gene expression levels is an inevitable process of plants responding to environmental stresses. Kasajima and Fujiwara first investigated high B-induced changes in gene expression in Arabidopsis thaliana roots and rosette leaves using microarray, and identified a number of high B-induced genes, including a heat shock protein and a number of the multi-drug and toxic compound extrusion (MATE) family transporters [5]. Hassan et al.
Results

Effects of B-toxicity on seedlings growth, B concentration in roots and leaves, and P concentration in leaves

Because B is phloem immobile in citrus plants, B-toxic symptoms first developed in old leaves. The typical visible symptom produced in B-toxic leaves was leaf burn (chlorotic and/or necrotic), which only occurred in *C. grandis* plants. In the later stages, B-toxic leaves shed prematurely. By contrast, almost no visible symptoms occurred in *C. sinensis* plants except for very few plants (Additional file 1).

B-toxicity-induced decreases in root, shoot and whole plant dry weights (DWs) were more pronounced in *C. grandis* than in *C. sinensis* seedlings (Figure 1A-C). Root DW decreased to a larger extent than shoot DW in response to B-toxicity, and resulted in a decrease in root DW/shoot DW ratio of both *C. grandis* and *C. sinensis* seedlings (Figure 1A-B and D).

B-toxicity increased B concentration in roots and leaves, especially in leaves and decreased P concentration in *C. grandis* leaves. No significant differences were found in root and leaf B concentration and leaf P concentration between the two species at each given B treatment except that B concentration was higher in B-toxic *C. sinensis* leaves than in B-toxic *C. grandis* ones (Figure 2).

Effects of B-toxicity on leaf gas exchange, pigments, total soluble protein and MDA

B-toxicity-induced decreases in both CO₂ assimilation and stomatal conductance were higher in *C. grandis* than in *C. sinensis* leaves. Intercellular CO₂ concentration increased in *C. grandis* leaves, but did not significantly change in *C. sinensis* leaves in response to B-toxicity. CO₂ assimilation and stomatal conductance in control leaves did not differ between the two species, but were higher in B-toxic *C. sinensis* leaves than in B-toxic *C. grandis* ones. Intercellular CO₂ concentration in control leaves was higher in *C. sinensis* than in *C. grandis*, but the reverse was the case in B-toxic leaves (Figure 3A-C).

B-toxicity decreased concentrations of Chl a+b and Car and ratio of Chl a/b in *C. grandis* and *C. sinensis* leaves. In control leaves, all the three parameters did not differ between the two species, but Chl a+b and Car concentrations were higher in B-toxic *C. sinensis* leaves than in B-toxic *C. grandis* ones (Figure 3E-G).

Leaf concentrations of total soluble protein and MDA were decreased and increased by B-toxicity in *C. grandis* leaves, respectively, but were not significantly affected in *C. sinensis* ones (Figure 3D and H).

B-toxicity-induced differentially expressed genes revealed by cDNA-AFLP

Here we used a total of 256 selective primer combinations to isolate the differentially expressed transcript-derived fragments (TDFs) from B-toxic leaves of two citrus species differing in B-tolerance. A representative picture of a silver-stained cDNA-AFLP gel showing B-toxicity-induced genes in *C. grandis* and *C. sinensis* leaves was presented in Additional file 2. As shown in Table 1, a total of 6050 clear
and unambiguous TDFs were detected from the B-toxic leaves, with an average of 25.7 (15–40) TDFs for each primer combination. Among these TDFs, 932 TDFs only presented in C. grandi, 631 TDFs only presented in C. sinensis, and 4587 TDFs presented in the two species.

A total of 218 and 104 differentially expressed and reproducible TDFs were successfully obtained from B-toxic C. grandis and C. sinensis leaves, respectively. All these TDFs were re-amplified, cloned and sequenced. For C. grandis, 183 of fragments yielded usable sequence data. Alignment analysis showed 132 TDFs were homologous to genes encoding known, putative predicted, uncharacterized, hypothetical or unnamed proteins, and the remaining 51 TDFs showed no significant matches (Tables 1 and 2). A total of 218 and 104 differentially expressed and reproducible TDFs were successfully obtained from B-toxic C. grandis and C. sinensis leaves, respectively. All these TDFs were re-amplified, cloned and sequenced. For C. grandis, 183 of fragments yielded usable sequence data. Alignment analysis showed 132 TDFs were homologous to genes encoding known, putative predicted, uncharacterized, hypothetical or unnamed proteins, and the remaining 51 TDFs showed no significant matches (Tables 1 and 2). Among these matched TDFs, 67 (50.8%) TDFs were up-regulated and 65 (49.2%) were down-regulated by B-toxicity. These TDFs were related to different biological processes such as cell transport (12.9%), lipid metabolism (2.3%), nucleic acid metabolism (12.9%), carbohydrate and energy metabolism (12.1%), protein and amino acid metabolism (25.0%), stress responses (6.1%), cell wall and cytoskeleton modification (6.8%), signal transduction (2.3%), other and unknown processes (16.2%) (Figure 4B).

Validation of cDNA-AFLP data using qRT-PCR
In this study, nine TDFs from C. sinensis leaves and nine TDFs from C. grandis ones were selected for qRT-PCR analysis in order to validate their expression patterns obtained by cDNA-AFLP analysis. Except for two TDFs (i.e., TDFs #187_1 and 195_1), the expression profiles of all the TDFs obtained by qRT-PCR were in agreement with the expression patterns produced by cDNA-AFLP (Figure 5). This technique was thus validated in 88.9% of cases. In addition to gene family complexity, the changes in the intensity of individual bands in the cDNA-AFLP gels might be responsible for the discrepancies between qRT-PCR and cDNA-AFLP analysis.

Discussion
C. sinensis displayed higher B-tolerance than C. grandis
Our results showed that the effects of B-toxicity on plant growth (Figure 1A-C), and leaf gas exchange, pigments, total soluble protein, MDA (Figure 3) and P (Figure 2C) were more pronounced in C. grandis than in C. sinensis seedlings, meaning that C. sinensis has higher B-tolerance than C. grandis. The present work, like that of the previous workers [8,13,15], indicates that the major of B in B-toxic citrus plants was accumulated in the control B-toxicity.

![Figure 1](image-url) **Effects of B-toxicity on growth of Citrus sinensis and C. grandis seedlings.** Bars represent means ± SE (n=10). (A-C) Root, shoot and root + shoot DWs. (D) Ratio of root DW to shoot DW. Bars represent means ± SE (n=10). Different letters above the bars indicate a significant difference at P <0.05.
leaves (Figure 2A and B). As shown in Figure 2B, B concentration was not lower in *C. sinensis* than in *C. grandis* leaves regardless of B concentration in the nutrient solution, indicating that *C. sinensis* leaves may tolerate higher level of B. Similar result has been obtained by Huang et al. [13]. Here we isolated 67 up-regulated and 65 down-regulated TDFs from B-toxic *C. grandis* leaves, whilst only 31 up-regulated and 37 down-regulated TDFs from B-toxic *C. sinensis* ones (Figure 4), suggesting that B-toxicity affects *C. sinensis* leaves gene expression less than *C. grandis* ones. These data also support above inference that *C. sinensis* leaves may tolerate higher level of B.

We found that CO₂ assimilation was lower in toxic leaves than in control leaves, while stomatal conductance was not lower in the former (Figure 3A-C), implies that B-toxicity-induced inhibition of CO₂ assimilation in two citrus species is primarily due to non-stomatal factors. Similar results have been obtained on B-toxic *C. grandis* and *C. sinensis* [13,14], ‘Navelina’ orange and ‘Clementine’ mandarin plants grafted on sour orange and Swingle citrumelo rootstocks [11,12], Newhall and Skagg’s Bonanza navel orange plants grafted on Carrizo citrange and trifoliate orange [9].

**Leaf carbohydrate and energy metabolism**

Since B-toxicity decreased CO₂ assimilation (Figure 3A), genes involved in photosynthesis and related biological processes might be affected by B-toxicity. As expected, 16 TDFs in *C. grandis* leaves and 14 TDFs in *C. sinensis* ones related to carbohydrate and energy metabolism were altered under B-toxicity (Table 2 and Figure 4). We found that B-toxicity decreased the transcript level of ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) small subunit precursor (TDF #143_2) gene in *C. grandis* leaves (Table 2), which agrees with the previous report that B-toxicity decreased the activity of Rubisco in *C. grandis* leaves [14]. Hudson et al. showed that the reduction of Rubisco concentration by anti-small subunit led to decreased photosynthesis in transgenic tobacco plants, but unchanged stomatal conductance [16]. Also, the mRNA abundances of photosystem II (PSII) 32 kDa protein (PsbA, TDF #251_1), chloroplast PSII oxygen-evolving complex 23 kDa polypeptide (TDF #112_2) and NifU-like protein (TDF #239_4) genes were down-regulated in B-toxic *C. grandis* leaves (Table 2). Khan et al. reported that PsbA knockout tobacco plants lacked PSII activity, accompanied by promoted senescence [17]. By using differential RNA interference (RNAi), Ishihara et al. demonstrated that PSII activity was linearly correlated with the total amount of PsbP (PSII 23 kDa protein) [18]. Ifuku et al. reported that PsbP is essential for the regulation and stabilization of PSII in higher plants [19]. Yabe et al. proposed that *Arabidopsis* chloroplastic NifU-like protein, which can act as a Fe-S cluster scaffold protein, was required for biogenesis of ferredoxin and photosystem I (PSI) [20]. B-toxicity-induced decreases in the transcript levels of PsbA, chloroplast PSII oxygen-evolving complex 23 kDa polypeptide and NifU-like protein genes agree with our report that B-toxicity impaired the whole photosynthetic electron transport from PSII donor side up to the reduction of end acceptors of PSI in *C. grandis* leaves [14]. By contrast, B-toxicity increased the transcript levels of chloroplast PSI oxygen-evolving complex 23 kDa polypeptide and NifU-like protein genes agree with our report that B-toxicity impaired the whole photosynthetic electron transport from PSI donor side up to the reduction of end acceptors of PSI in *C. grandis* leaves [14]. By contrast, B-toxicity increased the transcript levels of chloroplast PSI oxygen-evolving complex 23 kDa polypeptide (TDF #112_2) and glyceraldehyde-3-phosphate dehydrogenase B (TDE #23_2) in *C. sinensis* leaves (Table 2). NADP-glyceraldehyde-3-phosphate dehydrogenase is one of the two chloroplast enzymes...
Table 1 Summary of transcript-derived fragments (TDFs) from control and boron (B)-toxic leaves of *Citrus grandis* and *Citrus sinensis*

| Number of TDFs | Only present in *C. grandis* | Only present in *C. sinensis* | Present in both species | Total |
|----------------|-------------------------------|-------------------------------|------------------------|-------|
| Total TDFs detected from gels | 932                           | 631                           | 4487                   | 6050  |
| Total differentially expressed TDFs recovered from gels | 164                           | 50                            | 54                     | 268   |
| TDFs produced useable sequence data | 139                           | 46                            | 44                     | 229   |
| TDFs encoding known or putative proteins | 97                            | 40                            | 23                     | 160   |
| TDFs encoding predicted, uncharacterized, hypothetical or unnamed proteins | 9                             | 2                             | 3                      | 14    |
| TDFs without database matches | 33                            | 4                             | 18                     | 55    |
### Table 2 Homologies of differentially expressed cDNA-AFLP fragments with known gene sequences in database using BLASTN algorithm along their expression patterns in B-toxic leaves of *Citrus grandis* and *Citrus sinensis*

| TDF # | Size (bp) | Homology | Organism origin | E-value | Similarity (%) | Genebank ID | Ratio of BT/CK |
|-------|-----------|----------|-----------------|---------|----------------|-------------|---------------|
| 143_2 | 280       | Ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit precursor | *Citrus reticulata* | 6.00E-49 | 93%            | AAG49562.1 | 0             |
| 249_3 | 313       | Sedoheptulose-1 7-bisphosphatase | *M. truncatula* | 2.00E-48 | 97%            | XP_003600853.1 | +             |
| 223_2 | 253       | Glyceraldehyde-3-phosphate dehydrogenase B | *Arabidopsis thaliana* | 3.00E-06 | 84%            | NP_174996.1 | +             |
| 35_1  | 337       | 3-oxoacyl-reductase | *Zea mays* | 2.00E-05 | 85%            | NP_001167684.1 | 0             |
| 117_2 | 242       | Globular starch binding protein | *A. thaliana* | 8.00E-24 | 81%            | NP_568342.1 | 0             |
| 237_2 | 258       | 1,3-beta-D-glucanase GH17_65 | *Populus tremuloides* | 2.00E-31 | 78%            | NP_568377.1 | 0             |
| 239_4 | 223       | NifU-like protein | *Medicago truncatula* | 2.00E-32 | 77%            | NP_197423.1 | 0             |
| 23_2  | 253       | Starch branching enzyme I | *A. thaliana* | 1.00E-33 | 94%            | BAF01986.1 | 0             |
| 72_4  | 171       | Global transcription factor gro-A2 | *A. thaliana* | 2.00E-04 | 69%            | NP_192575.3 | 0             |

**Carbohydrate and energy metabolism**

| Homology | Organism origin | E-value | Similarity (%) | Genebank ID | Ratio of BT/CK |
|----------|-----------------|---------|----------------|-------------|---------------|
| CG       | CS              |         |                |             |               |

**Lipid metabolism**

| Homology | Organism origin | E-value | Similarity (%) | Genebank ID | Ratio of BT/CK |
|----------|-----------------|---------|----------------|-------------|---------------|
| CG       | CS              |         |                |             |               |

**Nucleic acid metabolism**

| Homology | Organism origin | E-value | Similarity (%) | Genebank ID | Ratio of BT/CK |
|----------|-----------------|---------|----------------|-------------|---------------|
| CG       | CS              |         |                |             |               |
Table 2 Homologies of differentially expressed cDNA-AFLP fragments with known gene sequences in database using BLASTN algorithm along their expression patterns in B-toxic leaves of *Citrus grandis* and *Citrus sinensis* (Continued)

| Fragment | Gene        | Species            | E-value | Identity (%) | Accession | gpm | fold change |
|----------|-------------|--------------------|---------|--------------|-----------|-----|-------------|
| 120_1    | IAA13       | Solanum lycopersicum | 3.00E-30 | 67%          | AE00356.1 |     | 3.3         |
| 44_1     | Elongator complex protein 3 | A. thaliana | 4.00E-53 | 89%          | NP_568725.1 |     | +           |
| 159_2    | Flowering time control protein FPA (AT2G43410) | A. thaliana | 0.14     | 32%          | BAH56948.1 |     | +           |
| 164_1    | ABA responsive element-binding protein | Solanum torvum | 3.00E-10 | 84%          | AFA37978.1 |     | 0           |
| 73_2     | Regulator of ribonuclease-like protein | M. truncatula | 2.00E-08 | 83%          | XP_003593378.1 |     | +           |
| 250_3    | RNA recognition motif-containing protein | A. thaliana | 7.00E-31 | 70%          | NP_563946.1 | 0.4 | 2.7         |
| 157_2    | RNA recognition motif-containing protein | A. thaliana | 3.00E-28 | 76%          | NP_188119.1 |     | 0           |
| 11_1     | Putative RNA helicase MTR4 | A. thaliana | 1.00E-44 | 82%          | NP_176185.1 |     | 0           |
| 71_3     | RNA helicase SDE3 | A. thaliana | 7.00E-24 | 71%          | AAK40099.1 |     | 0           |
| 186_1    | Chromodomain-helicase-DNA-binding protein | M. truncatula | 9.00E-56 | 73%          | XP_003625728.1 |     | 0           |
| 108_1    | Receptor for activated C kinase 1B | A. thaliana | 3.00E-40 | 87%          | NP_175296.1 |     | 0           |
| 67_4     | Sequence-specific DNA binding transcription factor | A. thaliana | 5.2     | 47%          | NP_566386.1 |     | 0           |
| 60_1     | AT5g24120/MLB8_4 | A. thaliana | 9.00E-37 | 63%          | AAK74018.1 |     | +           |
| 10_4     | GRAS family transcription factor | Populus trichocarpa | 2.00E-04 | 78%          | XP_002310226.1 |     | 0           |
| 22_3     | MAF1-like protein | Citrus sinensis | 2.00E-24 | 96%          | AVE43358.1 |     | 0           |
| 131_1    | RNA-binding (RRM/RBD/RNP motifs) family protein | A. thaliana | 7.00E-23 | 62%          | NP_171616.1 | 7.3 |           |
| 104_1    | Zinc finger CCCH domain-containing protein | M. truncatula | 1.00E-04 | 43%          | XP_003605843.1 |     | 0           |
| 68_2     | F14N23.20 | A. thaliana | 3.00E-27 | 83%          | AAD32882.1 |     | 0.3         |

**Protein and amino acid metabolism**

| Fragment | Gene                                | Species            | E-value | Identity (%) | Accession | gpm | fold change |
|----------|-------------------------------------|--------------------|---------|--------------|-----------|-----|-------------|
| 236_1    | Translation initiation factor IF-2, chloroplastic (AT1G17220) | A. thaliana | 4.00E-45 | 85%          | BAH20402.1 |     | 0           |
| 117_4    | Eukaryotic release factor 1-3 | Brassica oleracea var. botrytis | 3.00E-22 | 94%          | ACZ71035.1 |     | 0           |
| 93_3     | EMB1241 | A. lyrata subsp. lyrata | 5.00E-09 | 69%          | XP_002873846.1 | 0.4 |           |
| 73_3     | Ankyrin repeat domain-containing protein | M. truncatula | 5.00E-19 | 66%          | XP_003614004.1 | 0.2 |           |
| 179_4    | 50S ribosomal protein L15 | A. thaliana | 1.00E-18 | 80%          | NP_189221.1 |     | 0           |
| 105_1    | 30S ribosomal protein S17 | M. truncatula | 0.005    | 89%          | XP_00360547.1 | 0     | 0           |
| 99_6     | 60S ribosomal protein L6, putative | A. thaliana | 2.00E-18 | 93%          | AAM58875.1 |     | 0           |
| 186_2    | 60S ribosomal protein L4-1 | A. thaliana | 3.00E-52 | 90%          | NP_001030663.1 |     | 0           |
| 129_2    | 60S ribosomal protein L23 | A. thaliana | 2.00E-74 | 97%          | NP_001189805.1 |     | +           |
| 161_1    | 60S ribosomal protein L10B | Hevea brasiliensis | 3.00E-27 | 83%          | ADR71273.1 |     | +           |
| 93_2     | SHEPHERD | A. thaliana | 2.00E-26 | 86%          | BAB63688.1 |     | +           |
| 98_1     | Chaperonin 20 | A. thaliana | 2.00E-37 | 81%          | NP_197572.1 | 0     |             |
| 69_3     | AT5G47880 | A. thaliana | 3.00E-20 | 92%          | BAH19602.1 |     | 0           |
| 23_4     | MAP kinase | A. thaliana | 1.00E-20 | 98%          | CAB63149.1 |     | 0           |
| 139_4    | Putative leucine-rich repeat receptor-like protein kinase | A. thaliana | 4.00E-25 | 55%          | NP_209561.1 |     | 0           |
| 72_1     | CBL-interacting protein kinase 19 | Populus trichocarpa | 8.7     | 89%          | AB91226.1 |     | 0           |
| 39_3     | At1g25390/F2J7_14 | A. thaliana | 3.00E-23 | 81%          | AAK7715.1 |     | 0           |
| 12_2     | CDK activating kinase | Nicotiana tabacum | 3.7     | 46%          | BAF7582.4 |     | +           |
| 22_2     | Serine/threonine protein kinase ATR | M. truncatula | 6.00E-30 | 83%          | XP_003592675.1 |     | +           |
| 235_3    | Receptor-like protein kinase | M. truncatula | 9.00E-11 | 57%          | XP_00362121.1 | 0     | +           |
| 110_1    | Receptor-like protein kinase | A. thaliana | 2.00E-31 | 55%          | BAA9658.1 |     | 0           |
| 99_1     | Protein phosphatase 2C (PP2C) | Fagus sylvatica | 6.00E-30 | 71%          | CAB90633.1 | 2.6 | 3.7         |
| Entry | Accession | Description | Species | E-value | Identity | Description | Species |
|-------|------------|-------------|---------|---------|----------|-------------|---------|
| 99_2  | 273        | C3H4 type zinc finger protein | A. thaliana | 7.00E-28 | 64%      | NP_194986.2 | +       |
| 54_1  | 318        | AT5gS7360/MSF19_2 | A. thaliana | 1.00E-45 | 75%      | AAK64006.1 | +       |
| 57_1  | 246        | E3 ligase SAP5 | A. thaliana | 2.00E-37 | 84%      | NP_566429.1 | +       |
| 234_1 | 306        | Root phototropism protein 2 | A. thaliana | 9.00E-29 | 60%      | NP_001031446.1 | 2.8 3.4 |
| 96_1  | 229        | E3 ubiquitin-protein ligase BRE1-like protein | M. truncatula | 2.8 | 29% | XP_003637493.1 | 0       |
| 187_1 | 314        | Skp1-like protein 1 | Prunus avium | 4.00E-51 | 85% | AFJ21662.1 | 0       |
| 120_2 | 227        | Polyubiquitin | Vitis vinifera | 8.00E-39 | 100% | BAA76429.1 | 0       |
| 158_2 | 313        | Putative E3 ubiquitin-protein ligase XBAT31 isoform 2 | Vitis vinifera | 2.00E-18 | 63% | XP_002283974.1 | +       |
| 99_5  | 190        | Protein sodium-and lithium-tolerant 1 | A. thaliana | 1.00E-23 | 92% | NP_973625.1 | 0       |
| 104_3 | 171        | Transducin WD40 domain-containing protein (AtATG18a, AT3G62770) | A. thaliana | 3.00E-20 | 94% | NP_001030918.4 | 0       |
| 109_1 | 257        | Cold regulated 314 thylakoid membrane 2 | A. thaliana | 1.00E-19 | 56% | NP_564327.1 | 0       |
| 150_2 | 238        | Universal stress protein A-like protein | M. truncatula | 4.00E-27 | 71% | XP_003591417.1 | 0.2     |
| 38_4  | 261        | Serine carboxypeptidase II-3 | M. truncatula | 7.00E-21 | 74% | XP_003589243.1 | 0       |
| 2_1   | 276        | Group 5 late embryogenesis abundant protein (LEA5) | Citrus unshiu | 1.00E-35 | 94% | ABD93882.1 | 0       |

**Stress responses**

| Entry | Accession | Description | Species | E-value | Identity | Description | Species |
|-------|------------|-------------|---------|---------|----------|-------------|---------|
| 118_1 | 207        | Inorganic pyrophosphatase 1 | A. thaliana | 2.00E-16 | 83% | NP_565052.1 | 0       |
| 148_2 | 317        | Nudix hydrolase 19 | A. thaliana | 2.00E-48 | 78% | NP_197507.1 | 0 +     |
| 59_1  | 346        | Fe (II)/ascorbate oxidase family protein SRG1 | A. thaliana | 2.00E-16 | 71% | NP_173145.1 | 0       |
| 137_2 | 156        | Thioredoxin superfamily protein | A. thaliana | 3.00E-10 | 58% | NP_198706.1 | 0       |
| 68_3  | 146        | Thioredoxin superfamily protein | A. thaliana | 3.00E-07 | 59% | NP_201385.2 | 0.1     |
| 2_1   | 276        | Group 5 late embryogenesis abundant protein (LEA5) | Citrus unshiu | 1.00E-35 | 94% | ABD93882.1 | 0       |
| 125_1 | 389        | Thaumatin-like protein 1 | Apple tree | 9.00E-48 | 69% | JC7201 | +       |
| 99_5  | 190        | Protein sodium-and lithium-tolerant 1 | A. thaliana | 1.00E-23 | 92% | NP_973625.1 | 0       |
| 104_3 | 171        | Transducin WD40 domain-containing protein (AtATG18a, AT3G62770) | A. thaliana | 3.00E-20 | 94% | NP_001030918.4 | 0       |
| 109_1 | 257        | Cold regulated 314 thylakoid membrane 2 | A. thaliana | 1.00E-19 | 56% | NP_564327.1 | 0       |
| 150_2 | 238        | Universal stress protein A-like protein | M. truncatula | 4.00E-27 | 71% | XP_003591417.1 | 0.2     |

**Signal transduction**

| Entry | Accession | Description | Species | E-value | Identity | Description | Species |
|-------|------------|-------------|---------|---------|----------|-------------|---------|
| 182_2 | 117        | Signal recognition particle 54 kDa protein 2 | Solanum lycopersicum | 7.00E-07 | 93% | NP_001234428.1 | 0       |
| 108_2 | 257        | 14-3-3 protein | Dimocarpus longan | 6.00E-38 | 93% | ACK7623.1 | 0       |
| 200_1 | 240        | Heterotrimeric GTP-binding protein subunit beta 1 | Nicotiana tabacum | 3.00E-39 | 94% | AAG12330.1 | 0       |
| 70_2  | 252        | Pseudo-response regulator 5 | Castanea sativa | 5.00E-12 | 86% | ABV53464.1 | +       |
Table 2 Homologies of differentially expressed cDNA-AFLP fragments with known gene sequences in database using BLASTN algorithm along their expression patterns in B-toxic leaves of *Citrus grandis* and *Citrus sinensis* (Continued)

| Homologies                                                                 | Species         | E value | Identity | Accession Number | Log2 ratio |
|---------------------------------------------------------------------------|-----------------|---------|----------|------------------|------------|
| **Cell transport**                                                        |                 |         |           |                  |            |
| 26_1 342 H+-ATPase 6, plasma membrane-type                                | A. thaliana     | 1.00E-38| 97%      | NP_178762.1      | +          |
| 124_3 166 Calcium-transporting ATPase 1, endoplasmic reticulum-type      | A. thaliana     | 2.00E-14 | 83%     | NP_172559.1      | 3.1        |
| 66_1 177 Heavy metal ATPase                                              | P. trichocarpa  | 4.00E-15 | 78%     | XP_002303580.1   | +          |
| 97_1 201 Proton pump-interactor 1 (PPI1, AT4G27500)                      | A. thaliana     | 3.00E-12 | 56%     | BAH19433.1       | +          |
| 53_1 340 ABC transporter G family member 40                              | A. thaliana     | 9.00E-35 | 67%     | NP_173005.1      | +          |
| 210_1 247 Copper transporter                                             | P. trichocarpa  | 2.00E-15 | 64%     | XP_002298334.1   | +          |
| 178_1 297 Cyclic nucleotide-gated ion channel 1                          | A. thaliana     | 0.002   | 50%     | NP_200125.1      | +          |
| 49_3 252 Vacular-sorting receptor 3                                      | A. thaliana     | 1.00E-40 | 77%     | NP_179081.1      | +          |
| 137_1 249 Vacular protein-sorting-associated protein 37-1                | A. thaliana     | 0.48    | 63%     | NP_190880.1      | +          |
| 63_1 357 Vesicle-associated membrane protein-associated protein          | M. truncatula   | 3.00E-05 | 70%     | XP_003608721.1   | +          |
| 51_1 316 SecY protein transport family protein                           | A. thaliana     | 2.00E-51 | 87%     | NP_174222.5      | +          |
| 250_2 263 Fat-free-like protein                                          | M. truncatula   | 1.00E-32 | 82%     | XP_003591407.1   | +          |
| 79_2 237 Non-specific lipid-transfer protein                             | M. truncatula   | 1.00E-04 | 53%     | XP_003610781.1   | 2.5        |
| 67_3 268 Sieve element occlusion protein 1                               | Nicotiana tabacum | 6.00E-23 | 65% | AFN06072.1       | + +        |
| 89_2 230 AT5g24810/F6A4_20                                               | A. thaliana     | 1.00E-04 | 75%     | AAK82520.1       | 0          |
| 6_1 368 Protein transport protein SEC61 gamma subunit                     | Zea mays        | 2.00E-04 | 92%     | NP_001150911.1   | 0          |
| 249_2 370 Putative beta-subunit of adaptor protein complex 3, PAT2        | A. thaliana     | 2.00E-15 | 42%     | NP_567022.1      | 0 0        |
| 61_1 228 Sugar transporter ERD6-like 5                                    | A. thaliana     | 7.00E-15 | 57%     | NP_564665.3      | 0          |
| 179_2 225 Metal tolerance protein                                         | P. trichocarpa  | 6.00E-26 | 70%     | XP_00321066.1    | 0          |
| 51_4 221 Kinesin-related protein                                         | M. truncatula   | 0.38    | 35%     | XP_003612331.1   | +          |
| 36_2 319 Bidirectional sugar transporter SWEET7                          | A. thaliana     | 5.00E-08 | 60%     | NP_567366.1      | +          |
| **Cell wall and cytoskeleton modification**                              |                 |         |           |                  |            |
| 49_4 210 Caffeic acid 3-O-methyltransferase                               | M. truncatula   | 9.00E-23 | 68%     | XP_003602597.1   | 0          |
| 125_2 145 Caffeic acid O-methyltransferase 3                             | Gossypium hirsutum | 2.00E-05 | 55%     | ACZ06242.1       | 0.2        |
| 10_3 274 Chitinase                                                        | Citrus sinensis  | 3.00E-54 | 94%     | CA93847.1        | 0 0        |
| 249_4 217 Cellulose synthase                                              | Populus tremuloides | 1.00E-20 | 83%     | AAQ25581.1       | 0.2        |
| 33_3 249 O-methyltransferase 1                                            | A. thaliana     | 1.00E-33 | 74%     | AAB96879.1       | +          |
| 241_1 326 UMP domain-containing protein                                   | A. thaliana     | 1.00E-64 | 94%     | NP_195404.6      | +          |
| 124_2 385 UDP-glucose flavonoid 7-O-glucosyltransferase                   | M. truncatula   | 4.00E-12 | 73%     | XP_003629628.1   | +          |
| 3_3 225 UDP-glucosyltransferase family 1 protein                          | Citrus sinensis  | 6.00E-36 | 96%     | ACS87993.1       | +          |
| 70_4 176 Limonoid UDP-glucosyltransferase                                 | Citrus sinensis  | 2.00E-26 | 98%     | ACD14147.1       | +          |
| 63_2 228 Putative glucosyltransferase                                     | A. thaliana     | 2.00E-20 | 63%     | AAM61749.1       | 3.9        |
| **Other and unknown processes**                                           |                 |         |           |                  |            |
| 229_4 181 Phytoene synthase                                               | Citrus unshiu   | 1.00E-26 | 95%     | AAF33237.1       | 0          |
| 231_1 316 Strictosidine synthase family protein                           | A. thaliana     | 2.00E-28 | 68%     | NP_191262.2      | 0.4 2.6    |
| 72_3 194 Calcium-dependent lipid-binding domain-containing protein        | A. thaliana     | 8.00E-19 | 78%     | NP_564576.1      | + +        |
| 135_2 335 Oxidoreductase family protein                                   | Arabidopsis lyrata subsp.lyrata | 3.00E-40 | 65% | XP_002874584.1 | 0          |
| 5_2 262 Alkaline-phosphatase-like protein                                 | A. thaliana     | 7.00E-44 | 89%     | NP_194697.1      | 0          |
| 10_5 147 Protein tolB                                                     | M. truncatula   | 2.00E-06 | 55%     | XP_003630471.1   | +          |
which catalyze the reduction of 3-phosphoglycerate to triose phosphate [21]. However, the expression of Rubisco activase (TDF #6_4) gene in *C. sinensis* leaves decreased in response to B-toxicity (Table 2). Generally speaking, B-toxic *C. sinensis* leaves had higher expression levels of photosynthetic genes than B-toxic *C. grandis* ones. This might be responsible for the greater decrease in CO₂ assimilation in B-toxic *C. grandis* leaves compared with B-toxic *C. sinensis* ones. It is noteworthy that the mRNA level of gene encoding sedoheptulose-1,7-bisphosphatase (SBPase, TDF #249_3), a key factor for the RuBP regeneration, was up-regulated in B-toxic leaves of the two citrus species (Table 2). Harrison et al. showed that a small decrease in SBPase activity caused a decline in CO₂ assimilation by reducing the capacity for RuBP regeneration [22]. Lefebvre et al. observed that transgenic tobacco plants over-expressing SBPase had enhanced photosynthesis and growth from an early stage in development [23]. Wang reported that transgenic tomato plants over-expressing SBPase were more tolerance to low temperature and had higher photosynthetic capacity under low temperature [24]. Therefore, the up-regulation of SBPase might be an adaptive response to B-toxicity.

As shown in Table 2, B-toxicity decreased leaf expression levels of three genes [i.e., ADP-glucose pyrophosphorylase (TDF #235_2) in *C. sinensis*, starch branching enzyme I
plastids [25]. Thus, the fatty acid metabolism in B-toxic C. sinensis leaves might be impaired due to decreased plastidic pyruvate kinase.

In Arabidopsis, three NADPH: protochlorophyllide oxidoreductases (PORs), denoted as PORA, PORB, and PORC participate in mediating the light-dependent protochlorophyllide reduction [26]. Pattanayak and Tripathy showed that over-expression of PORC in Arabidopsis led to coordinated up-regulation of gene/protein expression of several Chl biosynthetic pathway enzymes, thus enhancing Chl synthesis, and that the $^{1}$O$_2$-mediated photo-oxidative damage in transgenic plants overexpressing PORC was minimal under high light stress [27]. The observed lower transcript level of PORC (TDF #171_2) in B-toxic C. grandis and C. sinensis leaves (Table 2) agrees with the results that B-toxicity decreased the concentration of Chl a + b in citrus leaves (Figure 3E).

Cytochrome P450s play a key role in biotic and abiotic stresses. Transgenic tobacco and potato plants expressing cytochrome P450 with increased monooxygenase activity tolerated better oxidative stress after herbicide treatment [28]. We found that B-toxicity increased the expression levels of genes encoding cytochrome P450 (TDF #5_1) and cytochrome P450 like protein (TDF #76-1) in C. grandis leaves (Table 2), which agrees with the previous report that some of the 49 cytochrome P450 genes in Arabidopsis were upregulated by biotic (i.e., Alternaria brassicicola and Alternaria alternata) and abiotic [i.e., drought, high salinity, low temperature, hormones, paraquat, rose bengal, UV stress (UV-C), mechanical wounding and heavy metal stress (CuSO$_4$)] stresses [29]. Thus, the up-regulation of cytochrome P450s in B-toxic C. grandis leaves might be an adaptive response. However, B-toxicity decreased the expression of cytochrome P450 in Arabidopsis roots [7].

 Taken all together, we isolated eight up-regulated and eight down-regulated TDFs from B-toxic C. grandis leaves, and five up-regulated and nine down-regulated from B-toxic C. sinensis ones. Among these differentially expressed TDFs, only SBPase (TDF #249_3) and PORC (TDF #171_2) were similarly affected by B-toxicity in the two species (Table 2). These results demonstrated that the transcript profiles in the two species were differentially altered under B-toxicity.

**Leaf lipid metabolism**

Allene oxide synthase (AOS) and hydroperoxide lyase (HPL) branches of the oxylipin pathway, which are responsible for the production of jasmonates and aldehydes, respectively, participate in a range of stresses. Recently, Liu et al. showed that depletion of rice OsHPL3 greatly stimulated the jasmonic acid-governed defense response [30]. Therefore, the AOS pathway and jasmonate level might be up-regulated in the B-toxic C. sinensis.

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**Figure 4 Functional classification of differentially expressed TDFs under B toxicity in Citrus grandis (A) and Citrus sinensis leaves (B).** Functional classification was performed based on the information reported for each sequence by The Gene Ontology (http://amigo1.geneontology.org/cgi-bin/amigo/blast.cgi) and Uniprot (http://www.uniprot.org/).

(TDF #42_1) in C. grandis and glucose-1-phosphate adenyllyltransferase large subunit 1 (TDF #59_2) in the two citrus species] related to starch biosynthesis, which agrees with the previous report that B-toxicity decreased starch concentration in C. grandis leaves [14].

B-toxicity increased the mRNA levels of three genes encoding citrate synthase (TDF #75-2), pyruvate dehydrogenase E1 component subunit beta (TDF #87_1) and aconitate hydratase 3 (TDF #33-2) in C. grandis leaves (Table 2), indicating that tricarboxylic acid cycle might be up-regulated in B-toxic C. grandis leaves. Similarly, the transcript level of a glycolysis gene encoding 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (TDF #161_3) was enhanced in B-toxic C. sinensis leaves (Table 2). However, the mRNA levels of plastidial pyruvate kinase 3 (TDF #35_1) and aconitate hydratase 1 (TDF #33_2) genes were reduced in B-toxic C. sinensis leaves (Table 2). There is evidence showing that plastidic pyruvate kinase plays a key role in fatty acid synthesis by controlling the supply of ATP and pyruvate for de novo fatty acid synthesis in
leaves due to decreased expression of fatty acid HPL (TDF #10_1; Table 2), thus contributing to B-tolerance. In addition, B-toxicity also affected the transcript levels of three genes [i.e., plastidial pyruvate kinase 3 (TDF #35_1), sugar-dependent1 (TDF #195_1) and 3-oxoacyl-reductase (TDF #233_3)] related to lipid metabolism in C. sinensis leaves (Table 2). Thus, lipid metabolism might be altered in B-toxic C. sinensis leaves.

Tang et al. reported that transgenic tobacco plants over-expressing acyl carrier protein (ACP)-1 (or expressing antisense ACP1) exhibited an increase (or decrease) in leaf concentrations of total lipids and the main fatty acids, and were more tolerant (or sensitive) to cold stress [31]. Branen et al. showed that reduction of ACP4 by antisense RNA led to a decrease in total leaf lipids and decreased photosynthetic efficiency, and concluded that ACP4 might play a major role in the biosynthesis of fatty acids for chloroplast membrane development [32]. The lower transcript level of gene encoding ACP1, chloroplastic-like (TDF #8_1) in B-toxic C. grandis leaves (Table 2) means that fatty acid biosynthesis in these leaves might be impaired. However, the expression of α/β-hydrolase domain-containing protein (TDF #194_1) and phospholipase-like protein (PEARLI 4) domain-containing protein (TDF #186_4) genes were up-regulated in B-toxic C. grandis leaves (Table 2).

Leaf nucleic acid metabolism

As shown in Table 2, eight up-regulated genes (TDFs #52_1, 49_1, 73_1, 120_1, 44_1, 159_2, 164_1 and 73_2) and nine down-regulated genes (TDFs #250_3, 157_2, 11_1, 71_3, 67_4, 10_4, 22_3, 104_1 and 68_2) were isolated from B-toxic C. grandis leaves, while only five up-regulated genes (TDFs #73_2, 104_2, 200_1, 107_1 and 59_1) and four down-regulated genes (TDFs #164_1, 71_3, 185_1 and 108_1) were identified in B-toxic C. sinensis leaves. Obviously, B-toxicity affected nucleic acid metabolism more in C. grandis leaves than in C. sinensis leaves.
ones. This agrees with our inference that *C. sinensis* may tolerate higher level of B.

**Leaf protein and amino acid metabolism**

All these differentially expressed TDFs encoding chloroplastic translation initiation factor IF-2 (TDF #236_1) involved in promoting the binding of forymethionyl-tRNA to 30 S ribosomal subunits, eukaryotic release factor 1–3 (TDF #117_4) involved in the termination step of protein synthesis, EMB1241 (At5g17710; TDF #93_3) related to protein folding and stabilization, Ankyrin repeat domain-containing protein (TDF #73_3) involved mainly in mediating protein-protein interactions, and ribosomal proteins [i.e., 50S ribosomal protein L15 (TDF #179_4), 30S ribosomal protein S17 (TDF #105_1), putative 60S ribosomal protein L6 (TDF #99_6) and 60S ribosomal protein L4_1 (TDF #186_2)] related to mature ribosome assembly and translation processes except for SHEPHERD (TDF #93_2) involved in the correct folding and/or complex formation of CLAVATA (CLV) proteins [33], 60S ribosomal protein L23 (TDF #129_2) and 60S ribosomal protein L10B (TDF #161_1), were down-regulated in B-tolerant *C. sinensis* leaves (Table 2), indicating that B-toxicity impairs protein biosynthesis in *C. grandis* leaves [34,35]. By contrast, only three down-regulated genes [30S ribosomal protein S17 (TDF #105_1), chaperonin 20 (TDF #98_1) involved in protein folding and stabilization and AT5G47880 (TDF #69_3) involved in the termination step of protein synthesis] were detected in B-tolerant *C. sinensis* leaves (Table 2). These results demonstrated that B-toxicity affected protein biosynthesis more in the former than in the latter. This agrees with our data that B-toxicity only decreased total soluble protein concentration in *C. grandis* leaves (Figure 3H).

Here we observed four down-regulated genes [i.e., mitogen-activated protein (MAP) kinase (TDF #23_4), putative leucine-rich repeat receptor-like protein kinase (TDF #139_4), CBL-interacting protein kinase 19 (TDF #72_1) and At1g25390/F2J7_14 (TDF #39_3)] and three up-regulated genes [i.e., CDK activating kinase (TDF #12_2), serine/threonine protein kinase ATR (TDF #22_2) and receptor-like protein kinase (TDF #235_3)] involved in phosphorylation and one up-regulated gene [i.e., protein phosphatase 2C (TDF #99_1)] involved in dephosphorylation in B-tolerant *C. grandis* leaves, while only one down-regulated gene [i.e., receptor-like protein kinase (TDF #110_1)] and one up-regulated gene [i.e., protein phosphatase 2C (TDF #99_1)] in B-tolerant *C. sinensis* leaves (Table 2). This means that *C. sinensis* leaves might achieve a better balance between phosphorylation and dephosphorylation than *C. grandis* ones under B-toxicity, which might contribute to the B-tolerance of *C. sinensis*.

Inactive (i.e., incorrect folding) and futile proteins for cell are tagged by ubiquitin for proteolysis [36]. In this study, we found four up-regulated genes [i.e., C3H4 type zinc finger protein (TDF #99_2), AT5g57360/MSF19_2 (TDF #54_1), E3 ligase SAP5 (TDF #57_1) and root phototropism protein 2 (TDF #234_1)] and three down-regulated genes [i.e., E3 ubiquitin-protein ligase BRE1-like protein (TDF #96_1), Skp1-like protein 1 (TDF #187_1) and polyubiquitin (TDF #120_2)] involved in ubiquitination in B-tolerant *C. grandis* leaves, and one up-regulated gene [i.e., putative E3 ubiquitin-protein ligase XBAT31 isoform 2 (TDF #158_2)] and two down-regulated genes [i.e., F-box family protein (TDF #73_1) and F-box with WD-40 2 (TDF #112_1)] involved in ubiquitination in B-toxic *C. sinensis* leaves. This indicates that ubiquitination might be involved in the adaptive response of citrus leaves to B-toxicity. Plant proteases has been shown to play key roles in controlling strict protein quality and degrading specific sets of proteins in response to environmental stresses [37]. As expected, several genes (TDFs #38_3, 81_1, 38_4, 73_4, 240_1, 39_1, 145_1, 67_1 and 75_1) involved in proteolysis were altered in B-tolerant *C. grandis* and *C. sinensis* leaves (Table 2).

S-adenosylmethionine (AdoMet) participates in a number of essential metabolic pathways in plants and is the principal biological methyl donor. AdoMet-dependent methylation is essential for keeping cellular functions in plants [38]. Methionine synthase, which catalyzes the last reaction in *de novo* methionine synthesis, also serves to regenerate the methyl group of AdoMet. As shown in Table 2, B-toxicity increased the expression of AT4G01850 (TDF #138_4) involved in AdoMet biosynthesis in *C. sinensis* leaves, but decreased *Methionine synthase* expression (TDF #245_1) in *C. grandis* leaves, which might contribute to the higher tolerance of *C. sinensis* leaves to B-toxicity than that of *C. grandis* ones.

N-carbamoylputrescine amidase (TDF #213_4) involved in polyamine (putrescine) biosynthesis were down-regulated in B-tolerant *C. grandis* leaves (Table 2). This means that the biosynthesis of polyamine might be inhibited in B-tolerant *C. grandis* leaves, which disagrees with the previous report that 1000 μM B increased leaf concentration of putrescine in B-sensitive barley cultivar, but decreased its concentration in B-tolerant one [39].

The up-regulation of 2-oxoglutarate-dependent dioxygenase gene (TDF #61_2) in B-tolerant *C. grandis* leaves (Table 2) agrees with the reports that B-toxicity stimulated the general amino acid control system in *Saccharomyces cerevisiae* [35] and that the concentration of total amino acids in tomato leaves increased under B-toxicity [40]. Evidence shows that 2-oxoglutarate-dependent dioxygenase participates in glucosinolate biosynthesis [41]. Thus, the concentration of glucosinolates might be enhanced in B-tolerant *C. grandis* leaves.
There is evidence showing that a few cystathionine-β-synthase (CBS) domain-containing proteins (CDCPs) play a role in plant stress response/tolerance and development [42]. Overexpression of OsCBSX4 improved tobacco plant tolerance to salinity, oxidative, and heavy metal stresses [43]. We observed that B-toxicity decreased the transcript level of CDCP (TDF #251_3) in C. sinensis leaves (Table 2), as obtained on manganese (Mn)-toxic C. grandis leaves [44]. However, B-deficient C. sinensis roots had higher level of CBS family protein [45]. Singh et al. observed that the expression of OsCBSX4 was up-regulated under high salinity, heavy metal, and oxidative stresses at seedling stage of a salt tolerant (Pokkali) rice cultivar, whilst its expression was upregulated only under NaCl stress, downregulated under heavy metal stress and kept unchanged under oxidative stress in a salt sensitive (IR64) rice one [43]. Taken all together, the influence of stresses on expression of CDCP genes depends on the kinds of stresses and plant species/cultivars.

**Leaf stress responses**

Inorganic pyrophosphatase (PPase), which leaves pyrophosphate molecules to liberate two molecules of inorganic phosphate, are essential for the viability of organisms, because the removal of pyrophosphate, a by-product of a host of biosynthetic reactions, is required for preventing the inhibition of thermodynamically unfavorable reactions [46,47]. George et al. observed that Nicotiana benthamiana plants lacking plastidial soluble PPase exhibited reduced drought tolerance as a result of the impaired leaf anabolic pathways [46]. The up-regulation of PPase (TDF #118_1) in B-toxic C. sinensis leaves (Table 2) might be an adaptive response to B-toxicity. By contrast, its expression (TDF #118_1) was down-regulated in B-toxic C. grandis leaves (Table 2).

Because leaf CO₂ assimilation was decreased in B-toxic leaves (Figure 3A), less of the absorbed light energy was utilized in photosynthetic electron transport in these leaves, particularly under high light. Thus, reactive oxygen species (ROS) production might be enhanced in B-toxic leaves because of more excess absorbed photon flux [14]. In addition to various ROS scavenger enzymes, “house-keeping” enzymes such as Nudix hydrolases (NUDXs) also play a role in ROS scavenging. Ogawa et al. [48] and Ishikawa et al. [49] showed that transgenic Arabidopsis plants overexpressing NURIDX2 and AtNURIDX7 exhibited higher tolerance to oxidative stress than wild type plants. Therefore, the higher expression level of NURIDX9 (TDF #148_2) in B-toxic C. sinensis leaves might be an adaptive response to B-toxicity (Table 2). However, its expression level (TDF #148_2) in C. grandis leaves decreased in response to B-toxicity (Table 2).

Up to 10% of the ascorbate content of the whole leaf is localized in the apoplast, where it forms the first line of defense against external oxidants [50]. In the apoplast, ascorbate oxidase (AO) oxidizes ascorbate to the unstable radical monodehydroascorbate which rapidly disproporionate to yield dehydroascorbate and ascorbate, thus participating in the regulation of the redox state of ascorbic acid pool. AO has been suggested to play a role in cell expansion via the modulation of redox control of the apoplastic (Table 51). Pignocchi et al. [52] showed that enhanced AO activity decreased the concentration and the redox state of ascorbic acid pool in the apoplast, whereas reduced AO activity increased its amount and redox state in the apoplast. Overexpression of AO in the apoplastic of tobacco resulted in lowered capacity for scavenging ROS in the leaf apoplast accompanied by increased sensitivity to ozone [53]. Fotopoulos et al. [50] observed that AO-overexpressing transgenic tobacco plants had increased sensitivity to various oxidative stress-promoting agents accompanied by a general suppression of the plant antioxidative metabolism. By contrast, a diminution in AO activity improved tomato yield under water deficit [54]. The down-regulation of gene encoding Fe (II)/ascorbate oxidase family protein SRG1 (TDF #59_1) in B-toxic C. sinensis leaves (Table 2) might increase the amount and the redox state of AA pool in the apoplast, thus enhancing the B-tolerance.

Thioredoxins, which participates in supplying reducing power to reductases required for detoxifying lipid hydroperoxides or repairing oxidized proteins, play key roles in plant tolerance of oxidative stress [55]. We found that the expression level of thioredoxin superfamily protein (TDF #137_2) was up-regulated in B-toxic C. grandis leaves (Table 2), indicating that thioredoxins might be involved in the ROS detoxification. However, the transcript level of thioredoxin superfamily protein (TDF #68_3) gene was down-regulated in B-toxic C. grandis leaves.

Our finding that B-toxicity increased the expression level of group 5 late embryogenesis abundant protein (LEA5, TDF #2_1) in C. grandis leaves (Table 2) agrees with the previous report that drought, heat and salt stresses stimulated the expression of LEA5 in citrus leaves [56]. Accumulation of ATRAB28 (LEA5) protein in Arabidopsis through transgenic approach improved the germination rate under standard conditions or salt and osmotic stresses and the cation toxicity tolerance [57]. Also, B-toxicity increased the transcript level of thaumatin-like protein 1 (TLPI, TDF #125_1) in C. grandis leaves (Table 2). The family of thaumatin-like proteins (also designated PR-5), which comprises proteins with various functions, is induced by biotic and abiotic factors in plants [58]. Therefore, the up-regulation of LEA5 and TLPI in B-toxic C. grandis leaves might be an adaptive response.

Protein sodium-and lithium-tolerant 1 (SLT1) gene isolated from tobacco (NtSLTI) and A. thaliana (AtSLTI)
has been implicated in mediating salt tolerance by regulating Na⁺ homeostasis via the calcineurin (CaN) and SPK1/HAL4 (SPK1/HAL4 which encodes a serine-threonine kinase) signal transduction [59]. Later, Antoine et al. [60] showed that rice OsSLT1 had molecular chaperone activity in vitro, and that OsSLT1 could be an important component of the cell immediate defenses against possible protein denaturation and aggregation. The down-regulation of SLTI (TDF #99_5) in B-toxic C. grandis leaves (Table 2) means that Na⁺ homeostasis or related processes mediated by SLTI are impaired in B-toxic C. grandis leaves.

Plant autophagy plays a role in various stress responses, pathogen defense, and senescence [61]. Xiong et al. [62,63] showed that AtATG18a was necessary for the formation of autophagosomes during nutrient stress and senescence in A. thaliana and that autophagy participated in the degradation of oxidized proteins under oxidative stress conditions in Arabidopsis. AtATG18a RNAi plants usually senescence earlier and have lower tolerance to various stresses including drought, salt and oxidative stresses compared with wild-type plants [61,63]. Our result showed that the transcript level of transducin WD40 domain-containing protein (ATG18a, TDF #104_3) in C. sinensis leaves decreased in response to B-toxicity (Table 2), indicating that autophagy is impaired in C. sinensis leaves.

As shown in Table 2, B-toxicity down-regulated the expression of “cold-regulated” gene (cold regulated 34 thylakoid membrane 2, TDF #109_1) in C. sinensis leaves and universal stress protein A-like protein (TDF #150_2) in C. grandis leaves (Table 2), indicating that B-toxicity might affect the tolerance of plants to other stresses.

Leaf signal transduction

Here four genes involved in signal transduction were altered by B-toxicity (Table 2 and Figure 4). Evidence shows that that signal recognition particle 54 kDa protein (SRP54) plays important roles in chloroplast development [64,65]. The down-regulation of signal recognition particle 54 kDa protein 2 (TDF #182_2) in B-toxic C. grandis leaves (Table 2) means that the biosynthesis of Chl is impaired in these leaves. This agrees with our results that B-toxicity affected Chl more in C. grandis leaves than in C. sinensis ones (Figure 3E).

Increasing evidence shows that 14-3-3 proteins play an important role in plant stress responses [66,67]. The most direct evidence for the role of 14-3-3 proteins in stress responses comes from transgenic rice plants over-expressing ZmGF14-6 encoding a maize 14-3-3 protein [68] and cotton plants over-expressing Arabidopsis 14-3-3A [69]. These transgenic plants displayed enhanced tolerance to drought stress. Heterotrimeric GTP-binding proteins (G proteins, consisting of subunits Gα, Gβ, and Gγ) are signaling molecules required for various eukaryotic organisms. Joo et al. [70] observed that A. thaliana mutant plants losing the Gβ protein were less tolerant to O₃ damage than wild-type plants. Thus, the B-tolerance of C. grandis leaves might be down-regulated due to decreased transcript level of genes encoding 14-3-3 protein (TDF #108_2) and heterotrimeric GTP-binding protein subunit beta 1 (TDF #200_1) (Table 2).

In higher plants, the endogenous circadian clock is involved in the manipulation of different various cellular processes ranging from photosynthesis to stress responses [71,72]. It also confers plants with competitive advantages, including improved photosynthesis, growth and survival [71]. Nakamichi et al. [72] observed that A PRR9, 7 and 5 triple mutant of Arabidopsis had higher tolerance against drought, salt and cold stresses compared to wild type, demonstrating the involvement of the three genes in abiotic stress responses as negative regulators. The up-regulation of pseudo-response regulator 5 (PRR5; TDF #70_2) in B-toxic C. sinensis leaves (Table 2) agrees with the previous reports that PRR5 was induced by cold treatment in apical shoots of cassava [73] and in Arabidopsis leaves [74]. Fukushima et al. [75] showed that PRR9, 7 and 5 negatively regulated the biosynthetic pathways of Chl, Car, ABA and α-tocopherol. This agrees with our results that B-toxic C. sinensis leaves had decreased concentrations of Chl a + b and Car (Figure 3E and H).

Leaf cell transport

As shown in Table 2 and Figure 4, the number of differentially expressed TDFs involved in cell transport was far less in B-toxic C. sinensis leaves than in B-toxic C. grandis ones, meaning that cell transport is less affected in the former than in the latter, which agrees with our inference that C. sinensis leaves may tolerate higher level of B.

Most of the differentially expressed TDFs (TDFs #26_1, 124_3, 66_1, 97_1, 53_1, 210_1, 178_1, 49_3, 137_1, 63_1, 51_1, 250_2, 79_2 and 67_3) associated with cell transport were up-regulated in B-toxic C. grandis leaves except for AT5g24810/F6A4_20 (TDF #89_2), protein transport protein SEC61 γ subunit (TDF #6_1) and putative β-subunit of adaptor protein complex 3, PAT2 (TDF #249_2) (Table 2), indicating that cell transport might be enhanced in B-toxic C. grandis leaves. Plasma-membrane H⁺-ATPase plays a crucial role in the plant response to environmental stresses, such as salt stress, aluminum (Al) stress, and potassium (K) deficiencies [76]. Wu et al. [77] reported that pumping of Ca²⁺ and Mn²⁺ by an endoplasmic reticulum-type Ca²⁺-ATPase (ECA1) into the endoplasmic reticulum was necessary for maintaining plant growth under calcium (Ca)-deficiency or Mn-toxicity. The PIB-ATPases (also known as heavy metal ATPases), which are involved in heavy metal transport across cellular membranes, play a crucial role in metal homeostasis and detoxification in
plants [78]. Proton pump interactor 1 (PPI1), an interactor of plasma-membrane H⁺-ATPase, stimulates its activity in vitro [79]. The up-regulation of PPI1 (TDF #97_1) in B-toxic leaves agrees with our data that the transcript level of H⁺-ATPase 6 (TDF #26_1) in C. grandis leaves increased in response to B-toxicity (Table 2) and with the report that the expression of PPI1 in potato tuber was up-regulated by salt stress and cold [79].

ATP-binding cassette (ABC) transporters are involved in metal ion efflux from the plasma-membrane. AtPDR8, an ABC transporter localized in the plasma-membrane of A. thaliana root hairs and epidermal cells, confers metal tolerance [80]. Our finding that the expression of ABC transporter G family member 40 (TDF #53_1) gene was up-regulated in B-toxic C. grandis leaves agrees with the reports that AtPDR8 in Arabidopsis roots and shoots was induced when exposed to copper (Cu), cadmium (Cd) and lead (Pb) [80], and that ABC transporter G family member 40 gene and ABC transporter A family member 7 gene were induced in drought-sensitive and -tolerant genotypes of Gossypium herbaceum, respectively under drought stress [81]. However, the expression of AT5g24810/F6A4_20 (TDF #89_2) was down-regulated in B-toxic C. grandis leaves (Table 2).

Cu transporters (COPTs/Ctrs) are involved in the maintenance of Cu homeostasis in plants. Generally speaking, COPTs/Ctrs are up-regulated by Cu deprivation and down-regulated by Cu excess [82]. COPT1 antisense Arabidopsis plants have decreased Cu level due to decreased Cu uptake and display sensitivity to Cu chelators [83]. The up-regulation of COPT (TDF #210_1) in B-toxic C. grandis leaves might play a role in the maintenance of leaf Cu homeostasis.

Plant cyclic nucleotide gated channels (CNGCs) play a role in heavy metal homeostasis. Previous study showed that transgenic tobacco plants overexpressing a truncated NtCBP4 (tobacco CNGC) had higher tolerance to Pb compared with wild type [84]. Chan et al. [85] reported that cngc2 Arabidopsis mutants were hypersensitive to increased soil Ca. However, transgenic tobacco plants overexpressing NtCBP4 were hypersensitivity to Pb [86]. B-toxicity-induced increase in transcript level of CNGC1 (TDF #178_1) in C. grandis leaves (Table 2) agrees with the report that the expression of AtCNGC2 was induced during Arabidopsis leaf senescence and AtCNGC2 might be involved in programmed cell death [87].

Membrane traffic is not only required for plant normal cellular function and maintenance of cellular viability, but also plays an important roles in plant responses to the environment [88,89]. The transcript levels of genes [i.e., vacuolar-sorting receptor 3 (TDF #49_3), vacuolar protein-sorting-associated protein 37–1 (TDF #137_1), vesicle-associated membrane protein-associated protein (TDF #63_1), secY protein transport family protein (TDF #51-1), fat-free-like protein (TDF #250_2) and non-specific lipid-transfer protein (TDF #79_2)] involved in membrane traffic increased in B-toxic C. grandis leaves except for genes encoding protein transport protein SEC61γ subunit (TDF #6_1) and putative β-subunit of adaptor protein complex 3, PAT2 (TDF #249_2) (Table 2). This indicates that membrane traffic might be enhanced in B-toxic C. grandis leaves.

Plant sieve element occlusion (SEO) genes have been shown to encode the common phloem proteins (P-proteins) that plug sieve plates after wounding. Tobacco SEO-RNA interference lines were essentially devoid of P-protein structures and lost photoassimilates more rapidly after injury than control plants [90]. Therefore, the up-regulation of sieve element occlusion protein 1 gene (TDF #67_3) in B-toxic C. grandis leaves (Table 2) might be of advantage to prevent the loss of photoassimilates. Recently, Huang et al. observed that many electron-dense particles deposited near sieve plates of B-toxic C. grandis and C. sinensis leaves [13]. In conclusion, the up-regulation of cell transport in B-toxic C. grandis leaves might be an adaptive response of plants to B-toxicity.

By contrast, we isolated three down-regulated [i.e., putative β-subunit of adaptor protein complex 3, PAT2 (TDF #249_2), sugar transporter ERD6-like 5 (TDF #61_1) and metal tolerance protein (MTP, TDF #179_2)] and three up-regulated [i.e., sieve element occlusion protein 1 (TDF #67_3), kinesin-related protein (TDF #51_4) and bidirectional sugar transporter SWEET7 (TDF #36_2) TDFs from B-toxic C. sinensis leaves (Table 2). Generally speaking, cell transport might not be enhanced in B-toxicity leaves.

In plants, kinesins are involved in a variety of cellular processes including intracellular transport, spindle assembly, phragmoplast assembly, chromosome motility, MAP kinase regulation and microtubule stability [91]. Li et al. [92] reported that mutation of rice BC12/GDD1 encoding a kinesin-like protein led to dwarfism with impaired cell elongation. Nishihama et al. [93] demonstrated that the expansion of the cell plate in tobacco plant cytokinesis required kinesin-like proteins (i.e., NACK1 and NACK2) to regulate the activity and localization of MAP kinase kinase. Therefore, the up-regulation of kinesin-like protein (TDF #51_4) in C. sinensis leaves (Table 2) might be an adaptive response to B-toxicity. However, the transcript level of putative β-subunit of adaptor protein complex 3, PAT2 (TDF #249_2) in C. sinensis leaves decreased in response to B-toxicity (Table 2).

Plant SWEETs function as facilitators involved in the influx and the efflux of sugar into and out of cells [94]. We found that the expression level of SWEET7 (TDF #36_2) in C. sinensis leaves increased in response to B-toxicity...
(Table 2), which agrees with the previous report that SWEET15/SAG29 was enhanced in senescing Arabidopsis leaves [95]. However, the expression of gene encoding sugar transporter ERD6-like 5 (TDF #61_1), a passive facilitator for the diffusion of glucose across the tonoplast membrane, was down-regulated in B-toxic C. sinensis leaves (Table 2). This disagrees with the previous report that the expression of AtESL1 (ERD six-like 1) was induced by various stresses including drought, high salinity and ABA in Arabidopsis plants [96].

MTPs are a subfamily of the cation diffusion facilitator (CDF) family found in plants. So far, most studied CDF family members confer heavy metal tolerance by affecting heavy metal efflux from the cytoplasm [97]. The down-regulation of MTP (TDF #179_2) in C. sinensis leaves (Table 2) means that the tolerance of plants to heavy metal might be reduced in B-toxic plants. This agrees with our previous report that the tolerance of C. grandis plants to Al-toxicity was higher under adequate B supply than under excess B [98].

Leaf cell wall and cytoskeleton modification

Eleven TDFs associated with cell wall and cytoskeleton modification were altered by B-toxicity (Table 2 and Figure 4). O-methyltransferase (OMT) genes are involved in lignin biosynthesis. Fu et al. [99] showed that down-regulation of the caffeic acid 3-O-methyltransferase (COMT) gene in switchgrass lowered lignin level in whole tillers and stems of transgenic plants and enhanced forage quality. Transgenic Leucaena leucocephala plants expressing antisense OMT displayed decreased activity of OMT activity and concentration of lignin [100]. Therefore, the biosynthesis of lignin in B-toxic C. grandis and C. sinensis leaves might be reduced due to decreased expression of COMT (TDF #49_4) and COMT3 (TDF #125_2) (Table 2). In addition, the biosynthesis of chitin in C. grandis and C. sinensis leaves and cellulose in C. grandis leaves might be down-regulated under B-toxicity due to the down-regulation of chitinase (TDF #10_3) and cellulose synthase (TDF #249_4) (Table 2). These results demonstrated that B-toxicity might impair citrus cell wall metabolism, which agrees with the previous suggestion that leaf cupping, a specific visible B-toxic symptom in some species might be due to the inhibition of cell wall expansion, through disturbance of cell wall crosslinks [101]. However, the transcript levels of genes encoding OMT1 (TDF #33_3), LIM domain-containing protein (TDF #241_1), UDP-glucose flavonoid 7-O-glucosyltransferase (TDF #124_2), UDP-glucosyltransferase family 1 protein (TDF #3_3), limonoid UDP-glucosyltransferase (TDF #70_4) and putative glucosyltransferase (TDF #63_2) in C. grandis increased in response to B-toxicity (Table 2).

Evidence shows that lily LIM1 [87] and all Arabidopsis LIM domain proteins [102] participate in regulating actin cytoskeleton organization and dynamics. Tobacco LIM1 protein acts in the cytoplasm as an actin binding and bundling protein [103] and in the nucleus as a transcription factor regulating the expression of genes related to lignin biosynthesis [104]. Recently, Moes et al. [105] demonstrated the involvement of tobacco LIM2 in actin-bundling and histone gene transcription. The up-regulation of LIM domain-containing protein (TDF #241_1) in B-toxic C. grandis leaves (Table 2) agrees with the report that the expression of LIM domain-containing protein in Physcomitrella patens increased under cold acclimation [106].

Glycosyltransferases (GTs), which catalyze the formation of glycosidic bonds between donor sugars and acceptor molecules, participate in many aspects of a plant life, including cell wall biosynthesis [107,108]. In Arabidopsis, up to 10 or 12 GT2 family members form the cellulose synthase catalytic subunit and callose synthase gene families [108]. In plants, UDP-glucosyltransferases (UGTs) have been suggested to play important roles in keeping cell homeostasis, regulating plant growth and improving their tolerance to environmental stresses [109]. Overexpression of UGT77E2 conferred tolerance to salinity and drought stresses in A. thaliana [110]. Transgenic tobacco plants overexpressing UGT78S5 exhibited enhanced salt tolerance [111]. Therefore, the up-regulation of UDP-glucose flavonoid 7-O-glycosyltransferase (TDF #124_2), UGT family 1 protein (TDF #3_3), limonoid UGT (TDF #70_4) and putative GT (TDF #63_2) genes in B-toxic C. grandis leaves (Table 2) might play a role in B-tolerance of plants. However, loss of function of a UGT73B2 alone or in conjunction with UGT73B1 and UGT73B3 resulted in enhanced oxidative stress tolerance in Arabidopsis, whilst transgenic Arabidopsis plants overexpressing UGT73B2 displayed decreased oxidative stress tolerance [112].

Others

Overexpression of bacterial or plant gene encoding phytoene synthase (PSY), a key regulatory enzyme in Car biosynthesis, led to enhanced level of total Car in various higher plants [113,114]. Transgenic Arabidopsis plants overexpressing PSY from euhalophyte Salicornia europaea had higher tolerance to salt stress than wild type plants by enhanced photosynthetic efficiency and antioxidative capacity [115]. Cidade et al. [116] showed that ectopic expression of PSY from Citrus paradisi fruit conferred abiotic stress tolerance in transgenic tobacco, which was correlated with the increased endogenous ABA level and expression of stress-responsive genes. Our finding that B-toxic C. grandis leaves had lower transcript of PSY (TDF #229_4; Table 2) means that the biosynthesis of Car and the antioxidative capacity may be decreased in B-toxic leaves. This agrees with our data that B-toxicity affected Car more in C. grandis leaves than in C. sinensis.
one (Figure 3G) and the inference that *C. grandis* may tolerate lower level of B.

Strictosidine synthase (Str), a key enzyme in alkaloid biosynthesis, catalyzes the condensation of tryptamine and secologanin leading to the synthesis of numerous monoterpenoid indole alkaloids in higher plants [117]. The up-regulation of Str family protein gene (TDF #231_1) in B-toxic *C. sinensis* leaves (Table 2) agrees with the previous report that Str in *Catharanthus roseus* leaves was enhanced under dehydration, salt and UV stresses [117] and that B-toxicity decreased IAA level in *Triticum durum* seedlings [118], because the expression of Str was inhibited by auxin [119]. B-toxicity-induced up-regulation of Str family protein gene (TDF #231_1) also agrees with our reference that the AOS pathway and jasmonate level might be up-regulated in the B-toxic *C. sinensis* leaves due to decreased expression of fatty acid HPL gene (TDF #10_1) (Table 2), because Str has been shown to be induced by jasmonate [120]. By contrast, the expression of Str family protein gene (TDF #231_1) was down-regulated in B-toxic *C. grandis* leaves (Table 2), which agrees with the previous report that cold stress led to Str down-regulation in *C. roseus* leaves [117].

**Conclusions**

B-toxicity affected *C. grandis* seedling growth, leaf CO₂ assimilation, pigments, total soluble protein, MDA and P more than *C. sinensis*, indicating that *C. sinensis* have higher B-tolerance than *C. grandis* ones. Under B-toxicity, *C. sinensis* leaves accumulated more B than *C. grandis* ones, meaning that the former may tolerate higher level of B. Using cDNA-AFLP, we successfully isolated 67 up-regulated and 65 down-regulated TDFs from B-toxic *C. grandis* leaves, whilst only 31 up-regulated and 37 down-regulated TDFs from B-toxic *C. sinensis* ones. This indicates that gene expression is less affected in B-toxic *C. sinensis* leaves than in *C. grandis* ones, which might be associated with the fact that *C. sinensis* leaves can tolerate higher level of B. The higher B-tolerance of *C. sinensis* might be related to the findings that B-toxic *C. sinensis* leaves had higher expression levels of genes involved in photosynthesis, which might contribute to the higher photosynthesis and light utilization and less excess light energy compared to the B-toxic *C. grandis* ones, and in ROS scavenging, thus preventing them from photo-oxidative damage. In addition, B-toxicity-induced alteration in the expression levels of genes encoding inorganic PPase 1, ATPG01850 and methionine synthase differed between the two species, which might also contribute to the B-tolerance of *C. sinensis*. In this study, a total of 174 differentially expressed TDFs were isolated from two citrus species, only 26 TDFs presented in the two citrus, the remaining TDFs presented only in *C. grandis* or *C. sinensis*, demonstrating that the B-toxicity-responsive genes differ between the two citrus species. For example, cell transport were up-regulated in B-toxic *C. grandis* leaves, whilst this did not occur in B-toxic *C. sinensis* leaves.

**Methods**

**Plant materials**

This study was conducted from February to December, 2011 at Fujian Agriculture and Forestry University. Plant culture and B treatments were performed according to Han et al. [14]. Briefly, 5-week-old uniform seedlings of ‘Xuegan’ (*Citrus sinensis*) and ‘Sour pummelo’ (*Citrus grandis*) were transplanted to 6 L pots containing fine river sand. Plants, two per pot, were grown in a greenhouse under natural photoperiod at Fujian Agriculture and Forestry University. Eight weeks after transplanting, each pot was supplied every other day until dripping with nutrient solution containing 10 μM (control) or 400 μM (B-toxic) H₃BO₃ and 6 mM KNO₃, 4 mM Ca (NO₃)₂, 2 mM NH₄H₂PO₄, 1 mM MgSO₄, 10 μM H₃BO₃, 2 μM MnCl₂, 2 μM ZnSO₄, 0.5 μM CuSO₄, 0.065 μM (NH₄)₆MoO₇O₂₄ and 20 μM Fe-EDTA for 15 weeks. At the end of the experiment, fully expanded leaves from different replicates and treatments were used for all the measurements. Leaves were collected at noon under full sun and immediately frozen in liquid nitrogen and were stored at −80°C until extraction.

**Measurements of plant DW, root and leaf B, leaf P, total soluble protein, MDA and pigments**

Ten plants per treatment from different pots were harvested and divided into their parts (roots and shoots). The plant parts were then dried at 75°C for 48 h and their DWs measured. B concentration in roots and leaves was assayed by ICP emission spectrometry after microwave digestion with HNO₃ [121]. Leaf P concentration was measured according to Ames [122]. Leaf total soluble protein was measured according to Bradford [123] using bovine serum albumin as standard after being extracted with 50 mM Na₂HPO₄-KH₂PO₄ (pH 7.0) and 5% (w/v) insoluble polyvinylpyrrolidone. Extraction and determination of leaf MDA were performed according to Hodges et al. [124]. Chl, Chl a, Chl b and Car were assayed according to Lichtenthaler [125] after being extracted with 80% (v/v) acetone.

**Measurements of leaf gas exchange**

Leaf gas exchange was measured using a CIARS-2 portable photosynthesis system (PP systems, Herts, UK) at ambient CO₂ concentration under a controlled light intensity of 990–1010 μmol m⁻² s⁻¹ between 9:00 and 11:00 on a clear day. During measuring, leaf temperature and air relative humidity were 32.2 ± 0.2°C and 66.6 ± 0.8%, respectively.
Leaf RNA extraction, cDNA synthesis and cDNA-AFLP analysis
Total RNA was extracted from ca. 300 mg of frozen mixed leaves from B-toxic and control plants of *C. grandis* and *C. sinensis* using Recalcitrant Plant Total RNA Extraction Kit (Centrifugal column type, Bioteke Corporation, China). There were three biological replicates for each treatment. Leave of 4–5 plants from different pots were mixed as a biological replicate. Equal amounts of leaves were collected from each plant. cDNA synthesis and cDNA-AFLP analysis were performed according to Zhou et al. [44].

Validation of cDNA-AFLP data using qRT-PCR
Total RNA was extracted from the frozen leaves as described above. qRT-PCR analysis was performed according to Zhou et al. [44]. Specific primers were designed from the sequences of 16 differentially expressed TDFs using Primer Primier Version 5.0 (PREMIER Biosoft International, CA, USA). The sequences of the F and R primers used were listed in Additional file 3. Samples for qRT-PCR were run in 3 biological replicates with 3 technical replicates. Leave of 4–5 plants from different pots were mixed as a biological replicate. Relative gene expression was calculated using ddCt algorithm. For the normalization of gene expression, citrus actin (GU911361.1) was used as an internal standard and the leaves from control plants were used as reference sample, which was set to 1.

Experimental design and statistical analysis
There were 20 pots (40 seedlings) per treatment in a completely randomized design. Experiments were performed with 3–10 replicates. Results represented the mean ± SE. Statistical analyses of data were carried out by ANOVA tests. Means were separated by the least significant difference test at *P* < 0.05 level.

Additional files

**Additional file 1:** Boron (B)-toxic symptoms on *Citrus grandis* and *Citrus sinensis* leaves. 1: Control leaves of *C. grandis*; 2: B-toxic leaves of *C. grandis*; 3: Control leaves of *C. sinensis*; 4: B-toxic leaves of *C. sinensis*.

**Additional file 2:** cDNA-AFLP profiles using one EcoR I selective primer and eight Mes I selective primers. One EcoR I selective primer: EcoR I-GG, Eight Mes I selective primers: Mes I-GT, GA, TC, TG, TT, TA, AC, and AG. 1: Control leaves of *Citrus grandis*; 2: B-toxicity leaves of *C. grandis*; 3: Control leaves of *Citrus sinensis*; 4: B-toxicity leaves of *C. sinensis*. Arrows indicate differentially expressed TDFs.

**Additional file 3:** Specific primer pairs used for qRT-PCR expression analysis.

**Abbreviations**
ABC: ATP-binding cassette; ACP: Acyl carrier protein; AdoMet: S-adenosylmethionine; AO: Ascorbate oxidase; AOS: Allene oxide synthase; B: Boron; Car: carotenoid; CBS: Cystathionine-β-synthase; CDCP: CBS domain-containing protein; CDF: Cation diffusion facilitator; cDNA-AFLP: cDNA-amplified fragment length polymorphism; Chl: Chlorophyll; CNGC: Cyclic nucleotide gated channel; COMT: Caffeic acid 3-O-methyltransferase; COPT: Cu transporters; DW: Dry weight; GT: Glycosyltransferase; HPL: Hydroperoxide lyase; IF: Initiation factor; LEAS: Group 5 late embryogenesis abundant protein; MAP: Mitogen-activated protein; MATE: Multi-drug and toxic compound extrusion; MDA: Malondialdehyde; MTP: Metal tolerance protein; NUDX: Nudix hydrolases; OMT: O-methyltransferase; POR: Proteochlorophyllide oxidoreductase; PPhase: Phosphophatase; PPII: Proton pump interactor 1; PPR: Pseudo-response regulator 5; PsbA: PSII 32 kDa protein; PsbP: PSII 23 kDa protein; Psi: Photosystem I; PSII: Photosystem II; PSY: Phytoene synthase; RNAi: RNA interference; ROS: Reactive oxygen species; Rubisco: RuBP carboxylase/oxygenase; RuBP: ribulose-1,5-bisphosphate; SBPase: Sedoheptulose-1,7-bisphosphatase; SEO: Sieve element occlusion; SLT1: Protein sodium-and lithium-tolerant 1; Str: Stricosidine synthase; TDF: Transcript-derived fragments; TLP: Thaumatin-like protein; UGT: UDP-glucosyltransferase.
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