

1-1-2019

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## Regulation of boron toxicity responses via glutathione-dependent detoxification pathways at biochemical and molecular levels in *Arabidopsis thaliana*

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Received: 08.05.2019 • Accepted/Published Online: 24.09.2019 • Final Version: 21.11.2019

**Abstract:** The fine-tuned regulation of the Halliwell–Asada cycle (ascorbate–glutathione pathway) in *Arabidopsis thaliana* under boron (B) toxicity was shown in our previous report. In this study, we investigated the expression levels of some members of the glutathione S-transferase (GST) superfamily, such as phi (*GSTF2*, *GSTF6*, *GSTF7*, and *GSTF8*), tau (*GSTU19*), and zeta (*GSTZ1*) classes in *Arabidopsis thaliana* that were exposed to 1 mM boric acid (1B) and 3 mM boric acid (3B). Additionally, the expression levels of genes for glutathione (GSH) and phytochelatin biosynthesis as well as miR169 and miR156 were evaluated in *Arabidopsis thaliana* exposed to 1B and 3B. Moreover, changes in the levels of total GST activity; GSH; and total, protein-bound, and nonprotein thiols were spectrophotometrically determined. GSH levels and nonprotein thiol content did not change significantly following both B-toxicity conditions. Expression levels of *GSH1* and *GSH2* stayed stable under 1B toxicity; however, *GSH1* expression increased significantly under 3B conditions in *Arabidopsis thaliana*. The expression levels of four genes from phi class members of GST were not dramatically changed under B-toxicity conditions. However, the transcript levels of miR169, *ATGSTU19*, and *ATGSTZ1* were significantly increased after 1B and 3B exposure. These GST genes may have a role in the dramatic increase of total GST activity under toxic B. To the best of our knowledge, this is the first report displaying an integrative view of high-B–induced regulation of GSH-dependent enzymatic machinery at different biological organization levels in *Arabidopsis thaliana*.

**Key words:** Gene expression, glutathione S-transferase, miRNA, plant, posttranscriptional regulation, toxic boron

### 1. Introduction

Although boron (B) is an essential micronutrient for plants (Warrington, 1923), high levels of B are a crucial problem for crops, mainly in arid areas in the world (Landi et al., 2012). Along with many physiological defects, a toxic level of B can give rise to oxidative stress (El-Shintinawy, 1999; Ardic et al., 2009; Kayihan et al., 2016) that usually triggers an antioxidative response in plants. Changes in regulation of the enzymatic and nonenzymatic antioxidants under B toxicity have been examined in many plant species. For example, increased levels of flavonoid and anthocyanin in tomato (Cervilla et al., 2012); higher phenolic content in sweet basil (Pardossi et al., 2015); significant increases in catalase activity (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.15.1.1), and ascorbate peroxidase (APX; EC 1.11.1.11) in chickpea (Ardic et al., 2009) and soybean (Hamurcu et al., 2013) were determined under toxic B conditions. In the ascorbate–glutathione cycle, APX reduces hydrogen peroxide ( $H_2O_2$ ) to  $H_2O$  by bringing about the peroxidation of ascorbate (AsA) and yielding

monodehydroascorbate radical. It is directly reduced to AsA or undergoes nonenzymatic disproportionation to AsA and dehydroascorbate (DHA). Then, DHA can be converted to AsA using reduced glutathione (GSH); then, oxidized glutathione (GSSG) is produced. Subsequently, glutathione reductase (GR) converts GSSG into GSH with NADPH. In particular, GSH is also used for phytochelatin (PC) synthesis as a GSH-derived peptide that participates in heavy metal detoxification in plants (Iannelli et al., 2002). In addition, glutathione-S-transferases (GST; E.C. 2.5.1.18) catalyze the conjugation of GSH to a wide variety of hydrophobic, electrophilic, and cytotoxic substrates. Plant GSTs can perform GSH-dependent reactions including peroxidation, isomerization, or oxidoreduction (Edwards and Dixon, 2005). They are classified into eight groups including phi (GSTF), tau (GSTU), lambda (GSTL), zeta (GSTZ), theta (GSTT), tetrachlorohydroquinone dehalogenase (TCHQD), dehydroascorbate reductase (DHAR), and an unclassified microsomal GST (Mohsenzadeh et al., 2011). Among them, classes of phi,

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tau, lambda, and DHAR are plant specific, whereas zeta and theta classes are also found in animals and fungi (Dixon et al., 1998). In all the regulations mentioned above, GSH, a low molecular weight compound and one of the important nonprotein thiols, is biosynthesized in two sequential ATP-dependent reactions and catalyzed by glutamate cysteine ligase (GSH1; EC. 6.3.2.2.) and glutathione synthetase (GSH2; EC. 6.3.2.3). Cysteine is used for GSH synthesis. By means of thiol metabolism, sulfur uptake and its assimilation culminate in cysteine synthesis. This metabolism and the AsA–GSH cycle play a critical role in metal toxicity tolerance by accumulating compatible organic compounds and scavenging reactive oxygen species (ROS) in plants (Singh et al., 2015).

Changes in nonenzymatic and enzymatic components of the AsA–GSH cycle under B toxicity were mostly studied at the biochemical level in plants such as barley (Karabal et al., 2003), tomato (Cervilla et al., 2007), and chickpea (Ardic et al., 2009). For this reason, in our previous report, we studied the enzymes of the AsA–GSH cycle at biochemical and transcriptional levels in order to gain better insight into the antioxidant machinery in *Arabidopsis thaliana* (Kayihan et al., 2016). Accordingly, in addition to strong stimulation of SOD, a fine-tuned regulation of the AsA–GSH cycle under B toxicity was suggested (Kayihan et al., 2016). Recently, microarray and proteomic analyses have shown that many genes related to the GST superfamily and GST proteins were induced under excess B conditions in barley (Öz et al., 2009), wheat (Kayihan et al., 2017), poplar (Yıldırım and Uylaş, 2016; Yıldırım, 2017), and *Arabidopsis* (Chen et al., 2014). However, until now, the changes in GSH synthesis and GSH-dependent detoxification pathways against toxic B have not been studied in plants. Thus, in this work, our aim was to assess toxic-B-responsive regulation of GSH metabolism at the biochemical, transcriptional, and posttranscriptional levels in *Arabidopsis thaliana*. First, changes in total GST activity; GSH; total, nonprotein, and protein-bound thiols; expression levels of some members of GST superfamily classes such as phi (*GSTF2*, *GSTF6*, *GSTF7*, and *GSTF8*), tau (*GSTU19*), and zeta (*GSTZ1*); and genes for GSH and phytochelatin biosynthesis such as glutamate cysteine ligase (*GSH1*), glutathione synthetase (*GSH2*), and phytochelatin synthase 1 (*PCS1*) were investigated in *Arabidopsis thaliana* exposed to toxic B. Second, in order to determine the transcriptional GSH regulation in response to high B in more detail, our goal was to find microRNAs (miRNAs) related to GSH-dependent detoxification pathways in *Arabidopsis thaliana*. We did not find any miRNAs targeting genes related to these pathways in *Arabidopsis thaliana*. Still, we aimed to determine expression levels of miR156 and miR169 under B-toxicity conditions because it was suggested that miR156

can play a role in cadmium detoxification and mediation by directing GST5 regulation in radish (Xu et al., 2013). In addition, miR169 targets GST in sugarcane and maize, and GST levels increase due to reduced levels of ssp-miR169 (Gentile et al., 2013, 2015). In fact, miR156 targets the Squamosa promoter-binding protein-like (SPL) family of transcription factors, and miR169 targets members of the NF-YA transcription factor in *Arabidopsis thaliana*.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

Seeds of *Arabidopsis thaliana* cv. Columbia were surface sterilized as explained in our previous report (Kayihan et al., 2016), and they were sown on MS medium (Murashige and Skoog, 1962) containing 100  $\mu$ M boric acid (control) and toxic levels of B (1 mM and 3 mM boric acid). Then, plates (15 seeds per plate) were cold-treated at 4 °C in the dark for 3 days and transferred to a controlled growth chamber (22  $\pm$  2 °C) with a 16-h light photoperiod with 300  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> and 50  $\pm$  5% relative humidity. Seedlings were grown for two weeks and then harvested for further analyses.

### 2.2. GST activity

In order to determine GST activity, total soluble proteins were first extracted from the seedlings, and their concentrations were determined according to the Bradford method (Bradford, 1976). The activity of GST was determined according to Habig et al. (1974). The reaction medium included 125 mM KPO<sub>4</sub> buffer (pH 7.8), 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) (10 mM stock prepared in 80% EtOH), and enzyme extract containing 300  $\mu$ g protein. The formation of S-2,4-dinitrophenyl glutathione, adduct of CDNB, was monitored by measuring the increase in absorbance at 340 nm for 2.5 min. A blank was reprepared for each sample to prevent spontaneous conjugation of GSH and CDNB, and an extinction coefficient of 9.6 mM<sup>-1</sup>cm<sup>-1</sup> was used to calculate GST activity, which was expressed as nmol/mg of product per minute.

### 2.3. GSH and protein-bound, nonprotein, and total thiols

The GSH level was determined according to Anderson (1985). Frozen seedlings were homogenized in 1.5 mL 5% (w/v) sulfosalicylic acid. The homogenate was centrifuged at 12,000 g for 20 min. Supernatant was used for subsequent analyses: 1 mL supernatant and 1 mL 100 mM K-PO<sub>4</sub> buffer (pH 7.0), including 0.5 mM Na<sub>2</sub>EDTA and 100  $\mu$ L 3 mM 5-5'-dithiobis-2-nitrobenzoic acid (DTNB), were added and thoroughly shaken. After 5 min, the absorbance was measured at 412 nm. It was expressed as absorbance per gram of fresh weight (FW).

In order to determine the level of nonprotein thiol (Del longo et al., 1993), 0.2 mL supernatant and 1 mL

100 mM phosphate buffer (pH 7.0) containing 0.5 mM Na<sub>2</sub>EDTA and 1 mL 3 mM DTNB were mixed and shaken thoroughly. After 10 min, the absorbance was measured at 412 nm. Nonprotein thiol concentration was calculated by using an extinction coefficient of 13,100 M<sup>-1</sup> cm<sup>-1</sup> and expressed as nmol/g FW.

The content of total thiol was determined as suggested by Cai et al. (2004). Frozen seedlings were ground and homogenized with 0.02 M EDTA and centrifuged at 16,260 g at 4 °C for 10 min. The supernatants (0.4 mL) were mixed with 0.2 M tris buffer (pH 8.2, 0.3 mL), 0.01 M DTNB (20 µL), and methanol (1.58 mL), and then they were incubated for 20 min in the dark at 22 °C. Absorbance was measured at 412 nm against a blank (without adding supernatant). Total thiol concentration was calculated by using an extinction coefficient of 13,100 M<sup>-1</sup> cm<sup>-1</sup> and expressed as nmol/g FW. Protein-bound thiol content was calculated by subtracting nonprotein thiol content from total thiols.

#### 2.4. Quantitative real-time PCR (qRT-PCR)

TRIzol reagent was used for total RNA isolation (Chomczynski and Sacchi, 1987) from *Arabidopsis thaliana* seedlings. Complementary DNAs (cDNAs) were synthesized from equal amounts of total RNAs (5 µg) (Thermo Scientific). The reaction mixture, conditions of qRT-PCR analyses, melting curves for gene expression, and technical details for miRNA expression [stem-loop qRT-PCR method according to Varkonyi-Gasic et al. (2007)] were similar to our previous report (Kayihan et al., 2016). Normalization was made using actin gene (*ACT2*) (Kayihan et al., 2016), and 2<sup>-deltaCt</sup> was used for the determination of fold change in each comparison. Sequence information for primers is shown in Table 1.

#### 2.5. Statistical analysis

All experiments were performed as four biological replicates (n = 4). One-way ANOVA and LSD test by SPSS statistical program were used to analyze the obtained data for GST activity; levels of GSH; and nonprotein, protein-bound, and total thiols. A *t*-test was used for qRT-PCR data. All data were presented as mean ± standard error of mean (SEM).

### 3. Results

#### 3.1. Responses of GSH metabolism to B toxicity at a biochemical level

Treatment 1B did not cause any significant change in total thiol content in *Arabidopsis thaliana* (Table 2). However, there was a significant increase in this content among *Arabidopsis* seedlings under 3B conditions, compared with control (C) and 1B. Similarly, the protein-bound thiol level stayed stable in response to 1B condition and increased significantly after 3B. The nonprotein thiol content and

GSH did not significantly change following 1B and 3B treatments. However, GST activity was greatly induced following both B-toxicity conditions, as compared to C. There were significant differences in GST activity between 1B and 3B treatments. In fact, when compared to control, four-fold and more than two-fold increases in this activity were determined after 1B and 3B treatments, respectively (Table 2).

#### 3.2. Responses of GSH metabolism to B toxicity at transcriptional and posttranscriptional levels

*GSH1* expression stayed unchanged in 1B and increased significantly (more than two-fold) in 3B (Figure 1). Toxic B conditions did not cause any significant change in *GSH2* expression when compared to the respective control. An increase in *PCS1* expression, although not a significant one, was observed solely under 1B conditions. However, both B toxicity treatments did not cause a significant difference in the expression levels of *GSTF2* and *GSTF8*. Furthermore, 1B did not affect the expression levels of *GSTF6* and *GSTF7*, whereas 3B led to a significant decrease in expression levels in *GSTF6* and *GSTF7*. In contrast to these members of phi class, the expression levels of *GSTU19* and *GSTZ1* genes were markedly induced under all toxic B treatments, compared to control. Notably, there were five-fold and almost four-fold increases in *GSTU19* expression in response to 1B and 3B treatments, respectively. *GSTZ1* expression was also induced three-fold and more than two-fold following 1B and 3B conditions, respectively (Figure 1).

Both toxic B conditions caused an increase in the expression levels of miR169 (Figure 2). Most significantly, 1B induced an almost four-fold increase. On the other hand, a slight but significant reduction in the expression levels of miR156 was found under both B toxicity conditions (Figure 2).

### 4. Discussion

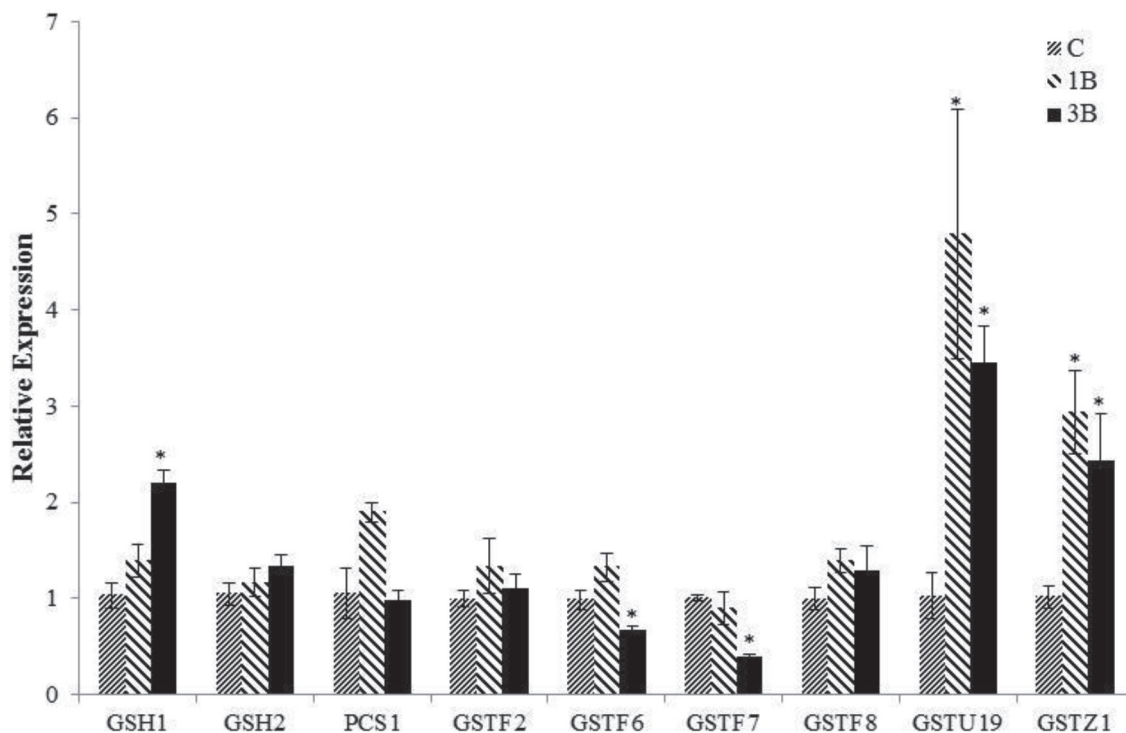
In our previous report, we have shown that oxidative damage is not provoked by higher B toxicity as phenolics and proline are promoted, SOD is strongly stimulated, and the components of the AsA–GSH cycle are coordinately regulated at the transcriptional level (Kayihan et al., 2016). Glutathione, an intermediate of the AsA–GSH cycle, is one of the most effective antioxidants in plant tissues, and it protects plant cells from abiotic stresses such as toxic metal stress by direct quenching of ROS, conjugation of toxic metals and other xenobiotics to GSTs, and acting as precursor for phytochelatin (PC) synthesis (Hasanuzzaman et al., 2017). Although the reduction in B accumulation via B transporters is the proposed mechanism of B tolerance, activation of the antioxidant system is also important for plants (Martinez-Cuenca et al., 2015). In this study, GSH levels did not significantly change in *Arabidopsis*

**Table 1.** The sequences of gene and miRNA-specific primers used for quantitative real time-polymerase chain reaction (qRT-PCR).

Primer name	Sequence 5' to 3'
GSH1_F	CAGTTCGAGCTTAGTGGTGC
GSH1_R	ATATCCTCCCGACGCCATTT
GSH2_F	CGTTTCCTGGCCTTAGTCGT
GSH2_R	CATGACTACCGCTCTTGGGT
GSTF2_F	AGTTTTTCGGACACCCAGCTT
GSTF2_R	TGGTCAAGCCGTAGATGGA
PCS1_F	CTCCTCCGGCCATTGACTTT
PCS1_R	ACCTCCAAGGCCCTTTCCAT
GSTF6_F	GCTTGGGTTGCTGACATCAC
GSTF6_R	TTCAAATCAAACACTCGGCAGC
GSTF7_F	ATCTTCCGCAACCCTTTTGGA
GSTF7_R	GGAGCCAAGGGAGACAAGT
GSTF8_F	GATCATCATGGCCAGTATCAAGG
GSTF8_R	GCTCTGACTCGAAAAGCGTC
GSTU19_F	GGGATGAGGACAAGGATCGC
GSTU19_R	CCTCTGAGCATCATAAGCTTCT
GSTZ1_F	ACCCTGAGCCACCTTTGTTA
GSTZ1_R	TAACCCAGGCAGTCTTCTCC
ACT2_F	CTTGACCTTGCTGGACGTGA
ACT2_R	AATTTCCCGCTCTGCTGTTG
miR156_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACGTGCTC
miR156_F	AGGCGGTGACAGAAGAGAGT
miR169_SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATAC GACTCGGCT
miR169_F	GATATGCAGCCAAGGATGACT
Universal_R	GTGCAGGGTCCGAGGT

**Table 2.** Changes in the levels of total, nonprotein, and protein-bound thiols; GSH; and total GST activity in leaves of *Arabidopsis thaliana*. C: control, 1B: 1 mM H<sub>3</sub>BO<sub>3</sub>, and 3B: 3 mM H<sub>3</sub>BO<sub>3</sub>. Values are means  $\pm$  SEM (n = 4). Values followed by different letters are significantly different at P  $\leq$  0.05. A: absorbance, FW: fresh weight.

Parameters	C	1B	3B
Total thiol (nmol g <sup>-1</sup> )	5.1 $\pm$ 0.2 <sup>a</sup>	5.3 $\pm$ 0.4 <sup>a</sup>	8.6 $\pm$ 0.4 <sup>b</sup>
Protein-bound thiol (nmol g <sup>-1</sup> )	4.57 $\pm$ 0.24 <sup>a</sup>	4.76 $\pm$ 0.35 <sup>a</sup>	7.84 $\pm$ 0.41 <sup>b</sup>
Nonprotein thiol (nmol g <sup>-1</sup> )	0.49 $\pm$ 0.04 <sup>a</sup>	0.58 $\pm$ 0.14 <sup>a</sup>	0.73 $\pm$ 0.045 <sup>a</sup>
GSH (A/gFW)	0.30 $\pm$ 0.003 <sup>a</sup>	0.28 $\pm$ 0.003 <sup>a</sup>	0.35 $\pm$ 0.039 <sup>a</sup>
GST activity (nmol min <sup>-1</sup> mg <sup>-1</sup> )	6.43 $\pm$ 0.32 <sup>a</sup>	25.43 $\pm$ 3.02 <sup>b</sup>	13.55 $\pm$ 0.05 <sup>c</sup>



**Figure 1.** Relative expression level of *GSH1*, *GSH2*, *PCS1*, *GSTF2*, *GSTF6*, *GSTF7*, *GSTF8*, *GSTU19*, and *GSTZ1* in response to toxic B treatments. C: control, 1B: 1 mM H<sub>3</sub>BO<sub>3</sub>, and 3B: 3 mM H<sub>3</sub>BO<sub>3</sub>. Values are means  $\pm$  SEM (n = 4). An asterisk above the bars represents significant differences between control and B-toxicity-treated samples ( $P \leq 0.05$ ).

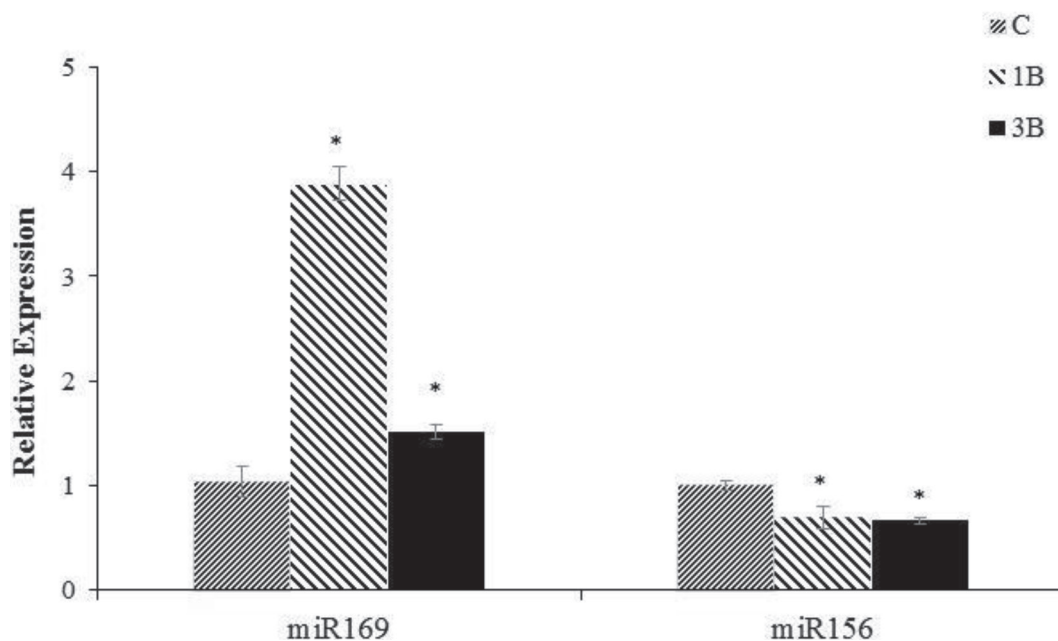
*thaliana* under 1B toxicity conditions. Coordinately, expression levels of *GSH1* and *GSH2* stayed stable in 1B. On the other hand, *GSH1* expression significantly increased under 3B condition, whereas *GSH* levels and *GSH2* expression remained unchanged. This means that upregulation of *GSH1* expression might not directly induce GSH levels, although it is the rate-limiting step of GSH biosynthesis (Noctor et al., 2012), because *GSH1* translational activation (Xiang and Bertrand, 2000) and posttranslational activation of *GSH1* (Jez et al., 2004) have already been suggested due to uncorrelated results between *GSH1* expression and its activity in *Arabidopsis thaliana* (May et al., 1998). Additionally, GSH is supplemented by regeneration from GSSG reduction that is catalyzed by GR in plant cells (Semane et al., 2007). In our previous report, toxic 3B conditions induced *GR1* and *GR2* expression but did not change GR activity (Kayihan et al., 2016). The function of GR, NADPH-dependent oxidoreductase, can be challenged by 3B treatment, indicating metabolic disruption due to the ability of B to bind with NADPH (Kayihan et al., 2016). This may be relevant to the stable GSH levels found under 3B conditions.

Nonprotein thiol content did not significantly change following 1B and 3B treatments. This may be related to GSH biosynthesis. As previously reported, an increase in GSH caused an increase in nonprotein thiol content,

which promoted heavy metal tolerance in maize (Requejo and Tena, 2012). Phytochelatin content might also be affected by stable GSH levels because they are synthesized from GSH. Correlatively, *PCS1* transcript was not notably changed under both B toxicity conditions. However, protein-bound and total thiol contents increased with increasing levels of toxic B.

The beginning of thiol metabolism is the uptake of sulfur and its successive assimilation (Wirtz and Hell, 2007). Supportively, genes (ta.6012.1.s1\_a\_at and ta.3736.1.a1\_x\_at) encoding for sulfate transmembrane transporter were differentially regulated in leaf and root tissues of a B-sensitive wheat cultivar under B-toxicity conditions (Kayihan et al., 2017). This means that some thiol-containing compounds may have a role in the B tolerance mechanism in plants.

The central position in the GSH network is occupied by GST (Labrou et al., 2015). Glutathione S-transferases play important roles in stress tolerance along with other protective functions in plants (Nianiou-Obeidat et al., 2017). In this work, a four-fold increase in GST activity was determined against 1B treatment. Interestingly, the change in this activity was lower under 3B conditions. This may be related to toxic-B-mediated oxidative stress, because a higher malondialdehyde (MDA) level was previously observed in 1B treatment as compared to 3B in *Arabidopsis*



**Figure 2.** Relative expression levels of miR169 and miR156 in response to toxic B treatments. C: control, 1B: 1 mM H<sub>3</sub>BO<sub>3</sub>, and 3B: 3 mM H<sub>3</sub>BO<sub>3</sub>. Values are means ± SEM (n = 4). An asterisk above the bars represents significant differences between control and B-toxicity-treated samples (P ≤ 0.05).

*thaliana* (Kayihan et al., 2016). Thus, it seems that total GST activity might dramatically increase in response to a sharp increase in MDA levels under 1B. Likewise, transgenic plants overexpressing one of the GST members suppress MDA concentrations under stress conditions (Xu et al., 2016; Gao et al., 2016; Xu et al., 2017). On the other hand, no correlation between GSH level and GST activity was found under B toxicity conditions. In other words, despite a stable level of GSH, a dramatic increase in GST activity may indicate undisturbed usage and recycling of GSH. Associatively, possible alternative pathways to convert GSSG back to GSH through class III peroxidases have been suggested due to uncorrelated results between GR and glutathione peroxidase in wheat (Liu et al., 2015).

Increased GST expression was shown to correlate with enhanced stress tolerance in tomato (Sun et al., 2010), barley (Rezaei et al., 2013), and wheat (Gallé et al., 2009). Copper treatment caused an increase in the expression levels of one tau and three phi class members of GST superfamily genes in *Arabidopsis thaliana* (Smith et al., 2004). Conversely, in this study, both B toxicity conditions did not cause a remarkable change in the expression levels of *GSTF2* and *GSTF8*, whereas 3B slightly reduced *GSTF6* and *GSTF7* expression. A slight reduction in *GSTF6* and *GSTF7* expression might be related to the lower increment of GST activity under 3B when compared to 1B conditions. In addition, it seems that the phi class of GST might not be involved in B tolerance or responsive mechanisms in

*Arabidopsis thaliana* exposed to toxic B. However, *GSTU19* was markedly induced following B toxicity conditions. Recently, it was reported that overexpression of *GSTU19* caused enhanced tolerance of salt, drought, and methyl viologen stresses in *Arabidopsis thaliana* as GST, and other antioxidant enzyme activities, and proline were increased, and the expression of some late stress-response genes was activated even under normal growth conditions (Xu et al., 2016). Likewise, *GSTU19* can be involved in the tolerance mechanism against B toxicity in *Arabidopsis thaliana*. Furthermore, *GSTZ1* was dramatically increased following both B toxicity conditions. *GSTZ1* gene is known for its role in tyrosine catabolism in *Arabidopsis thaliana* (Dixon et al., 2000). It seems that tyrosine catabolism might be affected by B toxicity in plants. Likewise, Gao et al. (2016) found by using *ThGSTZ1*-overexpressing transgenic *Arabidopsis thaliana* that *ThGSTZ1* regulated the activities and expression levels of protective enzymes and ROS scavenging ability and, thus, played a positive role in abscisic acid and methyl viologen tolerance.

In addition to locating toxic-B-responsive genes related to GSH metabolism, regulation of these genes is also important and may help to solve the underlying mechanism of B stress. Thus, we tried to find miRNAs related to GSH and/or GST in *Arabidopsis thaliana*. To date, among identified miRNAs, no GSH-related genes have been suggested as a target for miRNA in *Arabidopsis thaliana*. However, miR156 and miR169 have been

suggested to target GST5 in radish (Xu et al., 2013) and GSTU6 in *Zea mays* (Gentile et al., 2013). In *Arabidopsis thaliana* miR156 targets the Squamosa promoter-binding protein-like (SPL) family of transcription factors (Gandikota et al., 2007). Interestingly, through alignment analysis we found that the central site of miR156 is imperfectly complementary to the 3' UTR region of *GSTU19* in *Arabidopsis thaliana*. Repressed miR156 expression under toxic B conditions was correlated with upregulation of *GSTU19* expression. On the other hand, miR169 targets genes belonging to the nuclear factor Y family (NF-Y) transcription factor which has three distinct subunits (NF-YA, NF-YB, and NF-YC) binding to the CCAAT box in *Arabidopsis thaliana*. (Li et al., 2008). The miR169 expression increased most dramatically in 1B. This result suggests a positive correlation between miR169 expression and GST activity for B toxicity. This might be related to the fact that NF-YA regulates the expression of stress-responsive genes including GST (Li et al., 2008). Although it targets GSTU6 in *Zea mays*, we found that the seed region of miR169 is perfectly complementary to phi members of GST such as *GSTF2*, *GSTF7*, and *GSTF8*. This may reflect reduced or stable levels of expression of these genes under B toxicity conditions.

In our previous report, we found that the expression of *MDAR2* was coordinately regulated with *APX6* expression and total APX activity (Kayihan et al., 2016). These results were in accordance with our findings related to expression levels of miR169, *GSTU19*, *GSTZ1*, and total GST activity.

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- In conclusion, we suggest that *GSTU19* and *GSTZ1* might have roles in the dramatic increase of total GST activity under B toxicity conditions and that GST could play a special protective role in B toxicity tolerance in plants. Recently, Yildirim (2017) suggested a new internal B-detoxification mechanism resulting from higher upregulation of GST, HIPP, and ABC transporters in poplar exposed to toxic B. On the other hand, compartmentalization of B-anthocyanin complexes in vacuoles has been suggested as one of the tolerance mechanisms against B toxicity (Landi et al., 2015). Accordingly, anthocyanin-GSH or -GST complexes can transiently bind to metal or metalloid ions and, thus, form glutathionyl-anthocyanin-metal complexes, and/or GST-anthocyanin-metal complexes can be sequestered into the vacuole; GST-anthocyanin-metal complexes can also be exported by ABC transporters. We have already found that anthocyanin contents were significantly enhanced following B toxicity conditions (Kayihan et al., 2016), and ABC transporters are commonly and differentially upregulated in two contrasting wheat cultivars under high B (Kayihan et al., 2017). To summarize, our findings support an internal B detoxification mechanism via GSH-GST conjugation in plants. This information can be used for improving transgenic plants used for phytoremediation in contaminated soils with excess B.

## Acknowledgment

This study was supported by Gebze Technical University as a scientific research project (2016-A-12).



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