TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2021) 45: 181-191
© TÜBİTAK
doi:10.3906/bot-2101-36

The involvement of the induction of anthocyanin biosynthesis and transport in toxic boron responsive regulation in *Arabidopsis thaliana*

Ceyhun KAYIHAN*
Department of Molecular Biology and Genetics, Faculty of Science and Letters, Başkent University, Ankara, Turkey

Received: 26.01.2021 • **Accepted/Published Online:** 10.04.2021 • **Final Version:** 31.05.2021

Abstract: Recently, boron (B) has been found to form a complex with anthocyanin, which could be evidence for the B tolerance mechanisms that reduce free B in the leaf tissues of plants. However, the molecular mechanism of anthocyanin biosynthesis and transport has not been satisfactorily elucidated in plants exposed to toxic B. Therefore, the changes in expression levels of some of the phenylpropanoid pathway genes, early and late flavonoid biosynthetic genes, and transcription factors related to anthocyanin biosynthesis and transport were determined in *Arabidopsis thaliana* under B toxicity. Accordingly, 1 mM boric acid treatment did not cause any significant change in the expression levels of anthocyanin biosynthesis genes such as *PAL1*, *PAL2*, *C4H*, *4CL3*, *CHS*, *ANS* and transcription factors such as MYBD and TT8 in *Arabidopsis thaliana*. However, 3 mM boric acid treatment induced the expression levels of anthocyanin biosynthesis genes such as *C4H*, *4CL3* and transcription factors including MYB75, MYB114 and anthocyanin transporter genes such as *TT13* and *TT19*. In addition to B-anthocyanin, B-anthocyanins conjugated with glutathione (GSH) complexes can also participate in the internal B tolerance mechanism in plants. Therefore, the direct role of the B-anthocyanin complex without GSH conjugation needs to be determined. For this purpose, anthocyanin accumulation was determined in *slim1* mutant *Arabidopsis thaliana* exposed to excess B because SLIM1 transcription factor activates sulfate acquisition for S assimilation, which generates cysteine, the substrate for GSH. Accordingly, it was gradually increased through increasing toxic B levels in both wild type (WT) and *slim1* mutant plants. *slim1* mutant had more anthocyanin accumulation than WT under control and all toxic B conditions. In conclusion, increases in expression levels of MYB75, MYB114, *TT13*, TT19 and in anthocyanin level in *slim1* mutant in response to increased toxic B levels showed that anthocyanins may play a primary role in B tolerance in plants.

Key words: Anthocyanin biosynthesis, anthocyanin transport, *Arabidopsis thaliana*, boron toxicity, expression, SLIM1

1. Introduction

Boron (B) is an essential micronutrient for plant development and growth. B, which is slightly higher than its concentration for growth, can be toxic for plants (Mengel and Kirkby, 2001). It can easily accumulate in soil due to the poor drainage in arid and semiarid countries such as Morocco, Syria, Egypt, Iraq, Italy, and Turkey (Nable et al., 1997) and also, geothermal and volcanic processes and weathering are causes of excess B presence. Furthermore, evaporation from oceans contributes to B source (Landi et al., 2019). B toxicity causes limitation in crop yield and it affects the quality of product in many regions in the world (Aquea et al., 2012) because B accumulation at toxic levels in soil leads to impairment of growth and plant metabolism, causing chlorosis and necrosis in leaf tissues of plants (Reid et al., 2004; Landi et al., 2012). The uptake of excess B causes osmotic stress leading to inhibiting root growth (Aquea et al., 2012). In the presence of excess B, photo-oxidative stress is one of the reasons of plant growth inhibition (Reid et al., 2004); thus, oxidative stress might be caused by these physiological disorders due to overaccumulation of reactive oxygen species (ROS), causing cell death by oxidizing pigments, lipids, nucleic acids, proteins and inactivating enzymes (Blokhina et al., 2003). Plants have scavenging mechanisms including antioxidant enzymes against ROS accumulation. One of B tolerance mechanisms in plants is the enhancement of the antioxidant apparatus to combat B-triggered oxidative stress (Landi et al., 2012). Likewise, it was found that B toxicity caused an enhanced superoxide dismutase (SOD) expression and activity that resulted in H_2O_2 accumulation in *Arabidopsis thaliana* (Kayıhan et al., 2016).

Other proposed B tolerance mechanisms are the stimulation of synthesis of B-chelating organic compounds including polyalcohols (Papadakis et al., 2018) and phenolics (Landi et al., 2015), compartmentalization of B into vacuole (Wakuta et al., 2016) and cell wall (Papadakis et al., 2018) as well as reduction of uptake of B by root

* Correspondence: ckayihan@baskent.edu.tr

(Papadakis, 2016) and B efflux from the root (Reid, 2007). Within this context, it was surprisingly reported that anthocyanins have a role in ameliorating the detrimental effect of toxic B by forming B-anthocyanin complexes and favoring their shuttle into the vacuole (Landi et al., 2015; Landi, 2015) because chelating property of anthocyanin was thought to be only with metals; however, the same ability of anthocyanins was also shown for B as a metalloid (Landi et al., 2015; Pardossi et al., 2015; Estevez et al. 2021). The metal-chelating ability of anthocyanins has been suggested for Al, Cu, Fe, W, Cd, Ga, Zn, and Mo (reviewed by Landi et al. 2015). This chelating property decreases the free ion level in the plant cell, thereby alleviating detrimental effects of metal toxicity. For instance, it was found that Mo accumulation in leaves and stem was positively correlated with the content of anthocyanin in *Brassica rapa* exposed to excess Mo (Hale et al., 2001). Moreover, blue pigmentation of epidermal cells was observed in *Brassica rapa* exposed to W (Hale et al., 2002). Vacuolar sequestration of metal-anthocyanin-complexes in cell layers is a common protection mechanism by concentrating the toxic ions in nonphotosynthetic tissues of plants (Hale et al., 2002).

Similar to metal-anthocyanin complexation, B complexation with anthocyanin and reduction of free B in leaf tissues were postulated to be responsible for higher tolerance of purple-leafed than green-leafed basil cultivars exposed to toxic B (Landi et al., 2014; Estevez et al. 2021). Finally, anthocyanins may alleviate the metal or metalloid toxicity by forming anthocyanin–metal or metalloid toxicity complexes by relocating them to compartments or tissues where they are less harmful. However, until now, anthocyanin biosynthesis and transport have not been evaluated at molecular level under B toxicity conditions in plants. Thus, in this work, our first aim was to determine the transcript level of phenylpropanoid pathway genes such as phenylalanine ammonia lyase 1 (*PAL1*), phenylalanine ammonia-lyase 2 (*PAL2*), cinnamate 4-hydroxylase (*C4H*), and 4-coumaroyl: CoA-ligase (*4CL*) and early and late flavonoid biosynthetic genes such as chalcone synthase (*CHS*) and anthocyanidin synthase (*ANS*) and transcription factors related to anthocyanin biosynthesis such as MYB75, MYB114, and MYBD, and Transparent Testa 8 (TT8) and anthocyanin transport genes such as Transparent Testa 13 (*TT13*) and Transparent Testa 19 (*TT19*) in *Arabidopsis thaliana* following B toxicity treatments because beginning steps of the general phenylpropanoid pathway, early steps of the flavonoid pathway, and late steps of the anthocyanin specific pathway are three phases from phenylalanine to anthocyanins and *PAL1*, *PAL2*, *C4H*, *4CL*, *CHS*, and *ANS* are critical genes for these three steps for anthocyanin biosynthesis (Shi and Xie, 2014). Moreover, MYB75, MYB114, MYBD, and TT8 transcription factors are important for anthocyanin biosynthesis since expression of late biosynthetic genes of anthocyanins is mainly regulated by these transcription factors (Shi and Xie, 2014). Finally, anthocyanins need to be transported from the cytosol to the vacuole and *TT13* and *TT19* are required for transporter-mediated anthocyanin transport mechanism in plants (Kitamura et al., 2004; Shi and Xie, 2014).

Glutathione (GSH) also participates in vacuolar anthocyanin accumulation (Petrussa et al., 2013). Anthocyanins conjugated with GSH (alone or with Glutathione-S transferase (GST)) can transiently bind to metal or metalloid ions in the cytosol and form GSH-anthocyanin- or GST-anthocyanin-metal (or metalloid) complexes and these complexes can be sequestered into the vacuole by multidrug resistance-associated proteins and exported by ATP binding cassette transporters (Landi et al., 2015). Supportively, it was found that the expression levels of *GSTU19*, *GSTZ1*, and total GST activity were upregulated in *Arabidopsis thaliana* exposed to B toxicity (Kayıhan et al., 2019) and the ABC transporters were upregulated in two wheat cultivars with differing B tolerance under high B condition (Kayıhan et al., 2017). However, there is very limited information about the direct metal-chelating role of anthocyanin without GSH conjugation under B toxicity conditions in plants. Therefore, in this study, the level of anthocyanin accumulation in *slim1* mutant *Arabidopsis thaliana* was determined under toxic B conditions in order to understand the effect of GSH depletion condition (Yamaguchi et al., 2020) on anthocyanin accumulation because SLIM1 transcription factor activates sulfate acquisition for S assimilation, which is initiated by sulfate uptake, generates cysteine, the substrate for GSH (Yamaguchi et al., 2020). By this means, we can gain insight into the direct metal-chelating role of anthocyanin under limited GSH conjugation in plants. In conclusion, the determination of transcriptional regulation of genes related to anthocyanin biosynthesis and transport and transcription factors and also, anthocyanin accumulation under GSH depletion condition in *Arabidopsis thaliana* exposed to toxic B might provide more clear information about internal B tolerance mechanism in plants.

2. Materials and methods

2.1. Growth conditions and B toxicity treatment

Wild type (WT) (Columbia-0) seeds were kindly provided by Assistant Prof Dr Emre Aksoy from Niğde Ömer Halisdemir University. *slim1* (*slim1-1*) mutant *Arabidopsis thaliana* seeds were kindly provided by Associate Prof Dr Hideki Takahashi from Michigan State University with the help of Assistant Prof Dr Emre Aksoy. *slim1-1* mutant seeds were improved by mutagenizing the homozygous progeny of *PSULTR1;2-GFP* transgenic plants

(Columbia-0 background) (Maruyama-Nakashita et al., 2004) as a parental line with 0.3% ethyl methanesulfonate (Maruyama-Nakashita et al., 2006). They were surface sterilized as explained in our previous report (Kayıhan et al., 2016). WT seeds were directly sown into MS medium (Murashige and Skoog, 1962) including 100 µM H_3BO_3 (C), 1 mM H_3BO_3 (1B(D)), 2 mM H_3BO_3 (2B(D)), and 3 mM H_3BO_3 (3B(D)); however, *slim1* mutant seeds were directly sown into medium including C, 1B(D) and 2B(D) conditions because they did not tolerate the 3B(D) treatment. Five plates (each plate contained 15 seeds) for each treatment were cold treated at 4 °C in dark for 3 days, and then they were transferred to a controlled growth chamber (21 ± 2 °C) and a 16 h light photoperiod with 60% relative humidity. Moreover, in order to increase the toxic B concentration applied to *slim1* mutant *Arabidopsis thaliana*, 3B and 5B were applied to WT (for comparison) and *slim1* mutant *Arabidopsis thaliana* at seedling stage. In fact, they were grown under control condition for 11 days and were transferred to medium including 3 mM H_3BO_3 (3B(T)) and 5 mM H_3BO_3 (5B(T)) for 4 days.

2.2. Anthocyanin level

Fifteen-day-old WT *Arabidopsis thaliana* exposed to 1B(D), 2B(D), 3B(D), 3B(T), and 5B(T) and fifteen-day-old *slim1* mutant *Arabidopsis thaliana* exposed to 1B(D), 2B(D), 3B(T), and 5B(T) were harvested and used to determine the anthocyanin level according to Mancinelli et al. (1975). The seedlings were homogenized with 1 mL of extraction buffer (400 mL of 37% H_2O_2, 2.6 mL of dH_2O, and 12 mL of 100% methanol). They were transferred and incubated into tubes at 22 °C for 10 min. They were centrifuged at 15,000 × *g* for 5 min and supernatant was read at 530 and 657 nm. The following equation was used for anthocyanin content: $[A_{530}-(A_{657}/3)]/FW$.

2.3. Quantitative real-time PCR (qRT-PCR) conditions

WT *Arabidopsis thaliana* directly exposed to 1 mM H_3BO_3 (1B(D)) and 3 mM H_3BO_3 (3B(D)) were chosen for gene expression analyses because in our previous articles focusing on molecular mechanisms of toxic B in *Arabidopsis thaliana*, 1 mM B and 3 mM B were determined as moderate and severe levels of toxic B, respectively (Kayıhan et al. 2016, Kayıhan et al. 2019). Five micrograms of total RNA was isolated from leaf tissues of 15-day-old WT *Arabidopsis thaliana* according to Chomczynski and Sacchi (1987). The integrity of RNAs was determined by using agarose gel electrophoresis and also, the quality and quantity of RNA were determined by using NanoDrop spectrophotometer (Denovix, USA). One microgram of RNA was used to prepare first strand complementary DNA (cDNA) by using iScript cDNA Synthesis Kit (Bio-Rad). Every sample contained 1 µL of cDNA, 10 µL of iTaq universal SYBR Green super mix (2X) (Bio-Rad), 1 µL of forward primer (0.5 µM final concentration) and 1 µL of reverse primer (0.5 µM final concentration) and nuclease free water for a final volume of 20 µL. This mixture was used for qRT-PCR experiments (Roche LightCycler 480). Primers were designed from exon-exon boundary of the sequences of each gene by using NCBI database and they are shown in Table. Conditions of qRT-PCR were initial denaturation at 95 °C for 30 s, followed by 95 °C for 10 s, 59 °C for 1 min for 40 cycles. Conditions of melting curve were at 95 °C for 5s, at 65 °C for 1 min, and cooling for 40 °C for 30s. Expression levels of genes were normalized by using actin (*ACT2*) gene. The relative fold changes of expressions genes were calculated by $2^{-deltaCt}$.

2.4. Data statistics

All experiments were performed with at least three biological replicates. One-way ANOVA by SPSS statistical program was used to analyze the obtained data for anthocyanin levels. The data of qRT-PCR were statistically analyzed by using nonparametric versions of the t-test. They were presented as mean ± standard error of mean (SEM).

3. Results

3.1. Changes in anthocyanin levels in leaves of WT and *slim1* mutant *Arabidopsis thaliana* under B toxicity

The chlorosis was increased in leaves of WT and *slim1* mutant *Arabidopsis thaliana* through increasing toxic B levels (Figure 1). The leaf fresh weight of WT and *slim1* mutant *Arabidopsis thaliana* was gradually decreased in response to directly applied toxic boron conditions (1B(D), 2B(D)) (Figure 2). The leaf fresh weight of WT continued to decrease under 3B(D) condition. On the other hand, it was decreased under 3B(T) and 5B(T) conditions in WT and *slim1* mutant *Arabidopsis thaliana*; however, this decline was not as much as for direct applications (Figure 2). Moreover, the leaf fresh weight of *slim1* mutant *Arabidopsis thaliana* was decreased more than WT under control (C) (100 µM H_3BO_3) and all toxic B conditions (Figure 2).

The anthocyanin level was gradually increased through 1B(D), 2B(D), and 3B(D) conditions in leaves of WT *Arabidopsis thaliana* (Figure 3). Similarly, it was gradually increased through 1B(D) and 2B(D) conditions in leaves of *slim1* mutant *Arabidopsis thaliana* (Figure 3). In addition, it accumulated approximately three times more anthocyanins in *slim1* mutant *Arabidopsis thaliana* than WT under C condition. The highest increase of anthocyanin level was determined in response to 2B(D) treatment in *slim1* mutant *Arabidopsis thaliana* and also, in response to 3B(D) treatment in WT *Arabidopsis thaliana* (Figure 3). On the other hand, 3B(T) and 5B(T) treatments caused a gradual increase in anthocyanin level in both WT and *slim1* mutant *Arabidopsis thaliana* (Figure 3). This level is lower than the level determined

Table. Primer list of genes related to anthocyanin biosynthesis and transport and related transcription factors in *Arabidopsis thaliana*. F: Forward, R: reverse

AGI code	Gene name	Sequence 5' to 3'
AT1G65060 F	4-COUMARATE:COA LIGASE 3	AAAGGCTTTCAGGTCCCTCC
AT1G65060 R	4-COUMARATE:COA LIGASE 3	TCCATTTGATCGCACCACGA
AT1G56650 F	ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT	GGCTTCTAGGGAATAGGTGGTC
AT1G56650 R	ARABIDOPSIS THALIANA PRODUCTION OF ANTHOCYANIN PIGMENT	GGTGTTGTAGGAATGGGCGT
AT1G66380 F	MYB DOMAIN PROTEIN 114	GAGGGTTCGTCCAAAGGGTT
AT1G66380 R	MYB DOMAIN PROTEIN 114	CACCGATTTAGCCCAGCTCTT
AT1G70000 F	MYBD	CCAACCGATGACGGTGGTTA
AT1G70000 R	MYBD	ATGGAGTTCCTCGTTTGCGT
AT1G17260 F	TRANSPARENT TESTA 13	TGAACAAGTGTCATCCGCGA
AT1G17260 R	TRANSPARENT TESTA 13	TAGCCGCAAGTTGAGCAAGA
AT2G30490 F	CINNAMATE 4-HYDROXYLASE	TGAAGCTGAGCAGAAGGGAG
AT2G30490 R	CINNAMETE 4-HYDROXYLASE	AATGTTGTCTCAATCGCGGC
AT2G37040 F	PHENYLALANINE (PHE) AMMONIA LYASE 1	GAACTTATTAGATTCCTTAACGCCG
AT2G37040 R	PHE AMMONIA LYASE 1	AGTGTGGCAATGTGTGGCTT
AT3G53260 F	PHE AMMONIA LYASE 2	GAACTCATTAGATTTTTGAACGCCG
AT3G53260 R	PHE AMMONIA LYASE 2	ATTGCGGCAGTGTGTGACAT
AT4G09820 F	TRANSPARENT TESTA 8	TCCTCAACAACGGGTCTTGG
AT4G09820 R	TRANSPARENT TESTA 8	CTCCGCTGGTTGAGTTGTCT
AT4G22880 F	ANTHOCYANIDIN SYNTHASE	ACGCGAGTGGACAATTGGAA
AT4G22880 R	ANTHOCYANIDIN SYNTHASE	CGTACTCACTCGTTGCTTCTATGT
AT5G13930 F	CHALCONE SYNTHASE	TCAAGCGCATGTGCGACAA
AT5G13930 R	CHALCONE SYNTHASE	ACTTCGACCACCACGATGTC
AT5G17220 F	GLUTATHIONE S-TRANSEFERASE 26	TATGGACAGGTAACAGCAGC
AT5G17220 R	GLUTATHIONE S-TRANSEFERASE 26	AACTTGACCAAATGGCTGACG
AT3G18780 F	ACTIN 2	CTTGACCTTGCTGGACGTGA
AT3G18780 R	ACTIN 2	AATTTCCCGCTCTGCTGTTG

in *slim1* mutant *Arabidopsis thaliana* exposed to 2B(D) treatment. However, *slim1* mutant had more anthocyanin accumulation than WT under C and all toxic B conditions (Figure 3).

3.2. Changes in the expression levels of genes and transcription factors related to anthocyanin biosynthesis and transport in WT *Arabidopsis thaliana* under B toxicity

The expression levels of *PAL1* and *PAL2* were not significantly changed following 1B(D) and 3B(D) treatments in *Arabidopsis thaliana* (Figure 4). While 1B(D) treatment did not cause any significant change in the expression level of *C4H* gene, it was increased more than two-fold after 3B(D) (Figure 4). *4CL3* expression was not significantly changed following 1B(D) treatment in *Arabidopsis thaliana*; however, it was increased more than five-fold under 3B(D) treatment (Figure 4). However, there were no significant effects of 1B(D) and 3B(D) treatments on the transcript levels of *CHS* and *ANS* in *Arabidopsis thaliana* (Figure 4). MYB75 expression was significantly increased under both conditions in *Arabidopsis thaliana* (Figure 5). Especially, it was dramatically induced more than twelve-fold under 3B(D) condition (Figure 5). Likewise, MYB114 expression was slightly increased under 1B(D) treatment; however, it was dramatically induced (almost sixteen-fold) under 3B(D) treatment (Figure 5). Toxic B treatments did not result in statistically significant change in the expression level of MYBD in *Arabidopsis*

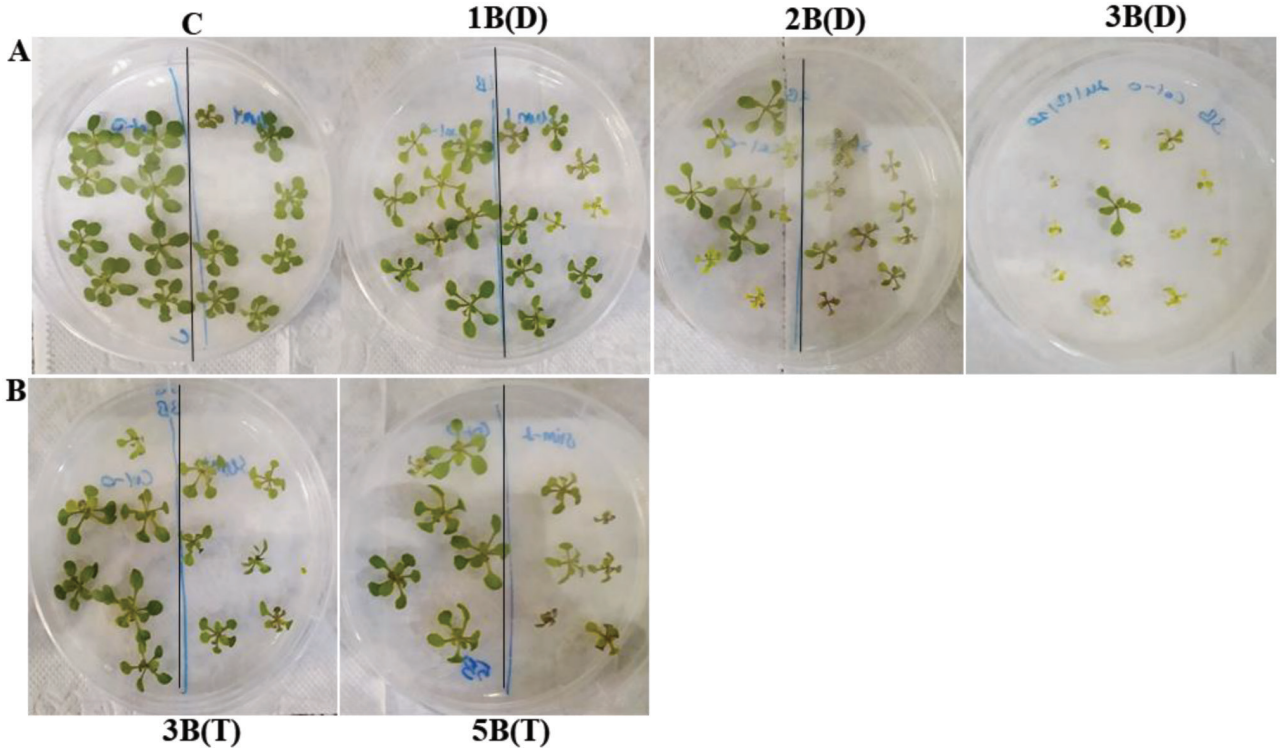

Figure 1. Fifteen-day-old WT and *slim1* mutant *Arabidopsis thaliana* seedlings exposed to control (C) and toxic B treatments. WT and *slim1* mutant *Arabidopsis thaliana* seeds were sown on the left and right sides of plates, respectively. A) WT seeds were directly sown into medium including 100 µM H_3BO_3 (C), 1 mM H_3BO_3 (1B(D)), 2 mM H_3BO_3 (2B(D)), and 3 mM H_3BO_3 (3B(D)); however, *slim1* mutant seeds were directly sown into medium including C, 1B(D), and 2B(D) conditions because they did not tolerate the 3B(D) treatment. B) In order to increase the toxic B concentration applied to *slim1* mutant *Arabidopsis thaliana*, 3B and 5B were applied to WT (for comparison) and *slim1* mutant *Arabidopsis thaliana* at seedling stage. In fact, they were grown under control condition for 11 days and were transferred to medium including 3 mM H_3BO_3 (3B(T)) and 5 mM H_3BO_3 (5B(T)) for 4 days.

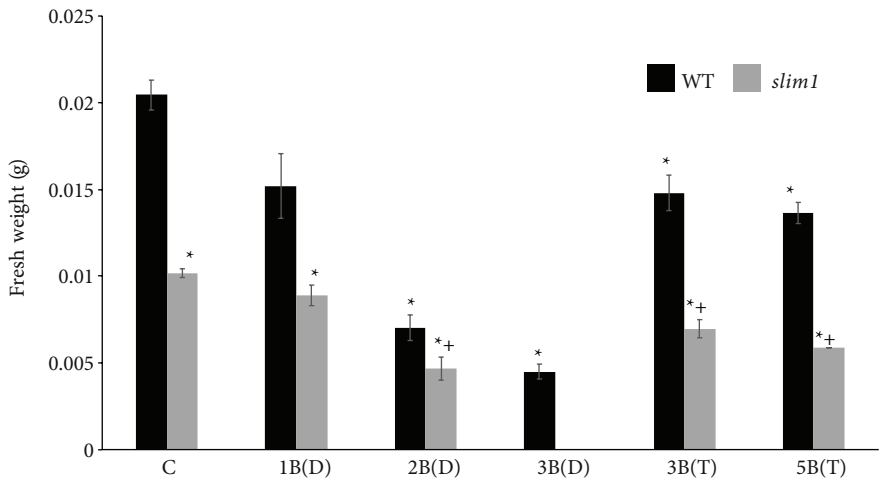

Figure 2. Changes in leaf fresh weight (g) of 15-day-old WT and *slim1* mutant *Arabidopsis thaliana* under different B conditions. C: Control, 1B(D): 1 mM H_3BO_3 applied directly to WT and *slim1* mutant *Arabidopsis thaliana* for 15 days, 2B(D): 2 mM H_3BO_3 applied directly to WT and *slim1* mutant *Arabidopsis thaliana* for 15 days, 3B(D): 3 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days 3B(T): Transferring of the WT and *slim1* mutant *Arabidopsis thaliana* grown under C condition for 11 days to 3 mM H_3BO_3 and exposing them to this medium for 4 days, 5B(T): Transferring of the WT and *slim1* mutant *Arabidopsis thaliana* grown under C condition for 11 days to 5 mM H_3BO_3 and exposing them to this medium for 4 days. Values are mean ± SEM (n = 4). Asterisks mean significant difference at $p < 0.05$ level according to C (WT) + indicates significant difference at $p < 0.05$ level according to *slim1* plant under C condition.

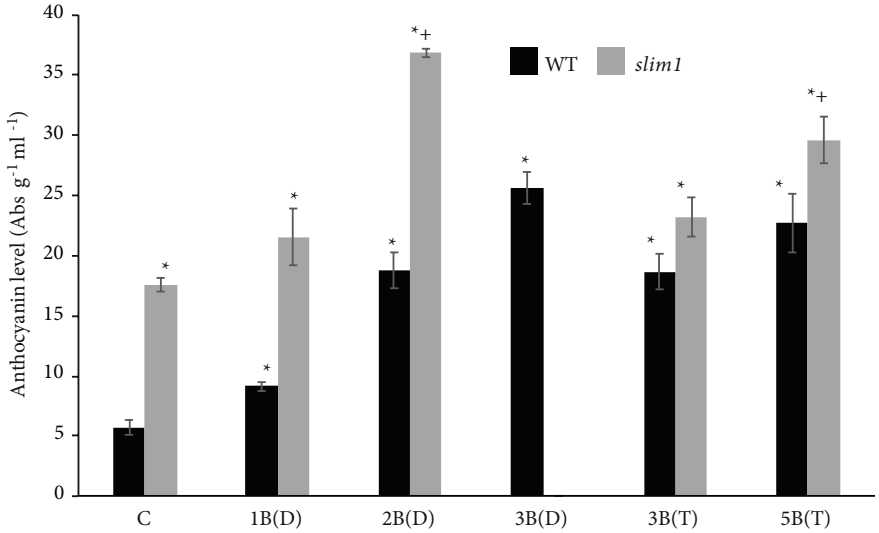

Figure 3. Changes in the anthocyanin level in leaf tissues of 15-day-old WT and *slim1* mutant *Arabidopsis thaliana* under different B conditions. C: Control, 1B(D): 1 mM H_3BO_3 applied directly to WT and *slim1* mutant *Arabidopsis thaliana* for 15 days, 2B(D): 2 mM H_3BO_3 applied directly to WT and *slim1* mutant *Arabidopsis thaliana* for 15 days, 3B(D): 3 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days 3B(T): Transferring of the WT and *slim1* mutant *Arabidopsis thaliana* grown under C condition for 11 days to 3 mM H_3BO_3 and exposing them to this medium for 4 days, 5B(T): Transferring of the WT and *slim1* mutant *Arabidopsis thaliana* grown under C condition for 11 days to 5 mM H_3BO_3 and exposing them to this medium for 4 days. Values are mean ± SEM (n = 4). Asterisks mean significant difference at $p < 0.05$ level according to C (WT) + indicates significant difference at $p < 0.05$ level according to *slim1* plant under C condition.

thaliana (Figure 5). Moreover, 1B(D) treatment did not cause statistically significant change in the transcript level of TT8, whereas it was upregulated more than two-fold after 3B(D) treatment (Figure 5).

The transcript levels of *TT13* gene were increased significantly in response to both toxic B conditions (Figure 6). However, 1B treatment did not lead to any significant change in the expression level of *TT19* gene (Figure 6). However, it was significantly increased almost seven-fold under 3B(D) condition (Figure 6).

4. Discussion

Reduced B accumulation in tissues was primarily suggested as B tolerance mechanism in plants (Nable, 1988). B transporters, which export excess B from plant tissues, are one of the well-known molecular mechanisms (Miwa and Fujiwara, 2011). Moreover, it has been recently suggested that free B reduction in plant tissues by B complexing with anthocyanins and their compartmentalization in vacuoles might provide B tolerance under toxic level of B (Landi et al., 2015). Supportively, in this study, anthocyanin accumulation was gradually increased through increasing toxic B conditions in WT *Arabidopsis thaliana*. As expected, severe B toxicity such as 3B(D), 3B(T), and 5B(T) treatments caused a dramatic increase in anthocyanin accumulation in WT *Arabidopsis thaliana*. On the other hand, GST and GSH also participate in vacuolar anthocyanin accumulation (Petrussa et al., 2013). Anthocyanins conjugated with GSH alone or through the activity of GST transiently bind to metal or metalloid ions in the cytosol, forming glutathionyl-anthocyanin-metal (or metalloid) complexes (Landi et al., 2015). Thus, GSH-deficient mutants of *Arabidopsis* were shown to be hypersensitive to different metals because of their inability to shunt metal ions into the vacuole or to deliver efficiently metal ions from the root to shoot tissues (Vernoux et al., 2000). However, the profile of anthocyanin accumulation has not been evaluated in GSH depleted plants under B toxicity in order to understand the importance of anthocyanin accumulation without GSH conjugation for metal or metalloid toxicity tolerance in plants. Therefore, in this study, the anthocyanin level in *slim1* mutant *Arabidopsis thaliana* exposed to toxic B conditions was determined in order to understand the effect of GSH depletion condition on anthocyanin accumulation because SLIM1 transcription factor activates sulfate acquisition for S assimilation, which is initiated by sulfate uptake, generates cysteine, the substrate for GSH by upregulating *SULTR1;1*, *SULTR1;2*, *SULTR4;1*, and *SULTR4;2* in response to S conditions (Yamaguchi et al., 2020). *SULTR1;1* and *SULTR1;2* facilitate the uptake of sulfate into roots and group 4 SULTR proteins are responsible for the efflux of sulfate from vacuoles (Yamaguchi et al., 2020). By this means, we can gain insight into the direct B-chelating role of anthocyanin under limited S and so, limited GSH conjugation in plants.

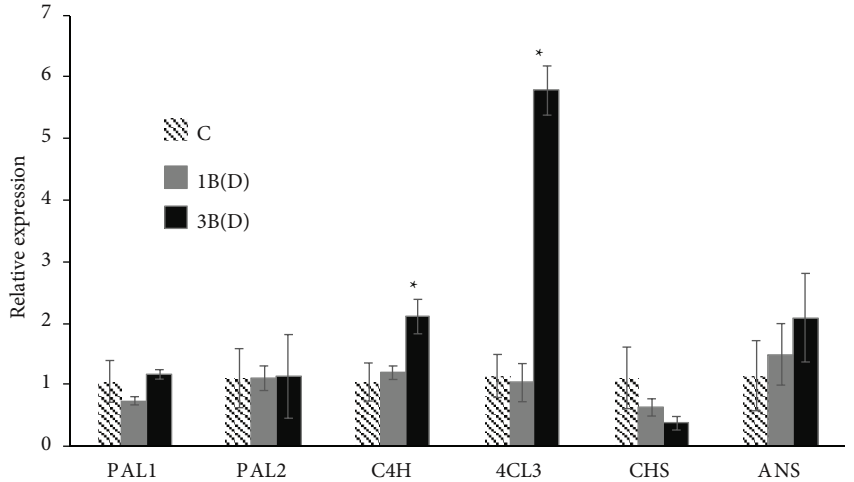

Figure 4. Changes in relative expression levels of the phenylpropanoid pathway genes and early and late flavonoid biosynthetic genes such as *PAL1*, *PAL2*, *C4H*, *4CL3*, *CHS*, and *ANS* in *Arabidopsis thaliana* (WT) in response to toxic B treatments. C: Control, 1B(D): 1 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days, 3B(D): 3 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days. The mean and SEM from three independent experiments are shown ($p < 0.05$). Asterisks mean significant difference at $p < 0.05$ level according to C.

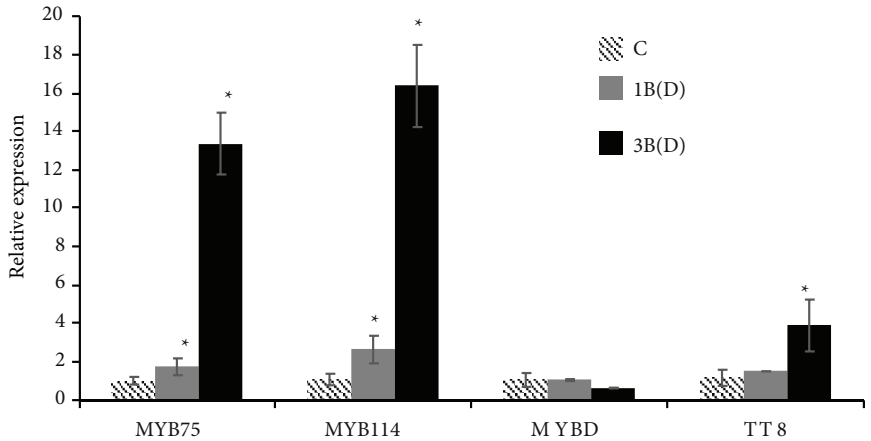

Figure 5. Changes in relative expression levels of transcription factors related to anthocyanin biosynthesis such as MYB75, MYB114, MYBD, and TT18 in *Arabidopsis thaliana* (WT) in response to toxic B treatments. C: Control, 1B(D): 1 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days, 3B(D): 3 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days. The mean and SEM from three independent experiments are shown ($p < 0.05$). Asterisks mean significant difference at $p < 0.05$ level according to C.

In this study, as expected, *slim1* mutant plant accumulated approximately three times more anthocyanins than WT plants under control condition because it was previously suggested that plants accumulate anthocyanin, alter their morphology, and exhibit earlier flowering under limited S condition (Nikiforova et al., 2004). On the other hand, all B toxicity treatments caused an increase in anthocyanin accumulation in WT and *slim1* mutant plants. It was gradually increased through increasing toxic B levels in both plants. This might be related to an increase in the B-chelating role of anthocyanin in response to an increased level of B toxicity in *Arabidopsis thaliana*. Moreover, anthocyanin accumulation was higher in *slim1* mutant than WT plant under all toxic B conditions. This might indicate the importance of anthocyanin accumulation for B-chelating under limited S or GSH depletion in *Arabidopsis thaliana*.

Although there are a few studies involving high-throughput gene expression analyses, transcriptional regulation of anthocyanin biosynthesis and transport has not been investigated in plants exposed to toxic B conditions. Thus, in this study, the changes in expression levels of genes involved in anthocyanin biosynthesis and transport and related transcription factors were determined in *Arabidopsis thaliana* because the biosynthesis of anthocyanin is primarily regulated at the transcriptional

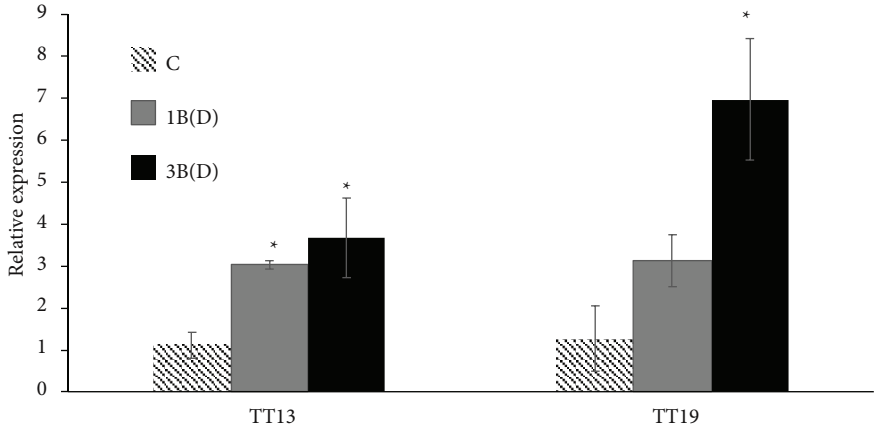

Figure 6. Changes in relative expression levels of anthocyanin transport genes such as *TT13* and *TT19* in *Arabidopsis thaliana* (WT) in response to toxic B treatments. C: Control, 1B(D): 1 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days, 3B(D): 3 mM H_3BO_3 applied directly to WT *Arabidopsis thaliana* for 15 days. The mean and SEM from three independent experiments are shown ($p < 0.05$). Asterisks mean significant difference at $p < 0.05$ level according to C.

level (Koes et al., 2005). The anthocyanin biosynthesis starts with phenylalanine (Phe). In general, beginning steps of phenylpropanoid pathway, early steps of flavonoid pathway, and late steps of specific anthocyanin pathway are three phases from phenylalanine to anthocyanins. PAL, C4H, and 4CL catalyze from Phe through cinnamic acid to 4-coumaroyl CoA in the first phase, respectively (Shi and Xie, 2014). The activity of PAL is modulated in response to environmental stresses in plants (Dixon and Paiva, 1995). In this study, the transcript levels of *PAL1* and *PAL2* were not significantly changed after 1B(D) and 3B(D) treatments in *Arabidopsis thaliana*. On the contrary, *PAL* gene expression was dramatically increased in leaf tissues of barley exposed to 5 mM B and 10 mM B (Öz et al., 2009). This might be related to complicated regulation of PAL activity at posttranslational and metabolic levels because the cellular PAL level increases transiently in response to environmental factors, and then it declines rapidly to basal levels, indicating rapid turnover of PAL enzyme (Shields et al., 1982; Jones, 1984). Moreover, a high level of intermediates of the biosynthetic pathway can cause feedback inhibition and this can trigger the rapid decay of PAL activity (Bubna et al., 2011). In this study, stable level of *PAL* expression might not indicate feedback inhibition of PAL in *Arabidopsis thaliana* exposed to B toxicity conditions because it was reported that downregulation of C4H resulted in a decrease in PAL activity in transgenic tobacco (Blount et al., 2000). However, we found that the transcript level of *C4H* was found to be increased significantly after 3B(D) treatment. In fact, the transcript level of *4CL3* genes were increased significantly after 3B(D) treatment. Likewise, *4CL* gene was significantly expressed under 10 mM B in barley (Öz et al., 2009) and it was increased five-fold in poplar under B toxicity condition (Yıldırım and Uylaş, 2016). Remarkable upregulation of *4CL3* expression caused by severe B toxicity can indicate the induced flavonoid pathway because it was found that that 4CL3 was preferably related to the flavonoid pathway (Ehlting et al., 1999), Supportively, flavonoid content was significantly increased in response to 1 mM B and continued to accumulate under 3 mM B in *Arabidopsis thalina* (Kayıhan et al., 2016).

The second phase of biosynthetic pathway (early steps) is from 4-coumaroyl CoA through chalcone to dihydroflavonol. 4-coumaroyl CoA and three molecules of malonyl-CoA yield a naringenin chalcone by CHS (Shi and Xie, 2014). In this study, transcript level of *CHS* did not significantly change under both toxic B conditions in *Arabidopsis thaliana*. This might be related to transcriptional and posttranslational regulations of CHS because it is inhibited noncompetitively by products of flavonoid pathway such as naringenin (Hinderer and Seitz, 1985). They accumulated in cytosol to a level which inhibits the activity of CHS to protect plant from toxic level (Whitehead and Dixon, 1983). In other words, CHS exhibits a transient de novo enzyme synthesis and the decay of CHS enzyme under abiotic stress conditions (Chappell and Hahlbrock, 1984; Ryder et al., 1984). This CHS induction was regulated by transient increase of *CHS* transcripts under stress conditions and the inactivation or degradation of CHS proteins caused the decline of its activity (Schröder and Schäfer, 1980).

As mentioned above, anthocyanin biosynthesis is primarily regulated at the transcript level because R2R3-MYB, bHLH, and WD40-repeat transcription factors and their interactions mainly determine the expression patterns of the anthocyanin structural genes by binding specific cis-acting elements in promoter of these genes (Koes et al., 2005; Lang et al., 2010). MYB75, MYB114, and the bHLH TT8 are involved in upregulation of

anthocyanin synthesis in *Arabidopsis* leaves by controlling transcript level of late biosynthesis genes such as *ANS* and dihydroflavonol reductase (*DFR*) (Gonzalez et al., 2008; Dubos et al., 2010). In this work, MYB75 and MYB114 were gradually increased through 1B(D) to 3B(D) treatments. These results are consistent with the gradual increase in anthocyanin accumulation under the same conditions. However, a trend of increase in *ANS* expression was observed but, interestingly, it was not significant. This might be related to dynamic regulation of transcription factors and their targets such as *ANS* and posttranslational regulation for anthocyanin biosynthesis in *Arabidopsis thaliana* under B toxicity condition because production of anthocyanins is spatio-temporally regulated in plants (Gou et al., 2011). Moreover, uncoordinated results might be related to complex regulatory mechanisms of anthocyanin biosynthesis and transport in plants because Poustka et al., (2007) found a dramatic increase in anthocyanin-filled subvacuolar structures without change in the levels of total anthocyanin after inhibition ATP-dependent transporters and they proposed an alternative mechanism of vesicular transport and vacuolar sequestration of anthocyanins in *Arabidopsis thaliana*.

The anthocyanin biosynthesis occurs in the cytosol and they are required to be transported from the cytosol to the vacuole (Shi and Xie, 2014). In *Arabidopsis*, *TT13* and *TT19* have functionally been characterized to be related to the anthocyanins transport (Shi and Xie, 2014). *TT13* has a proton pump function in the endothelium cells of seed coat and creates the driving force for TT12-mediated transport of proanthocyanidins precursors to the vacuole (Appelhagen et al., 2011). The vacuolar uptake mechanism of anthocyanins remains unclear in vegetative tissues (Shi and Xie, 2014). In this work, the transcript level of *TT13* gene was increased significantly under both toxic B conditions. It might also be a potential candidate for anthocyanin transport in vegetative tissues under toxic B conditions. On the other hand, *TT19* expression was significantly increased almost seven-fold under 3B(D) condition. It functions as a carrier protein to escort anthocyanins and/or precursors of proanthocyanidin to the vacuole, providing cyanidin protection from degradation during the transport process (Kitamura et al., 2004). Hence, severe B toxicity might induce the transporter-mediated anthocyanin import into vacuole in *Arabidopsis thaliana*.

5. Conclusion

Compartmentalization of B-anthocyanin complexes in vacuoles has been suggested as a tolerance mechanism 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 ABC transporters are commonly and differentially upregulated in two contrasting wheat cultivars under high B (Kayıhan et al., 2017) and also, GST can play a special protective role in B toxicity tolerance in plants (Kayıhan et al., 2019). In this study, the direct B-chelating role of anthocyanin under limited S and so, limited GSH conjugation was determined in *Arabidopsis thaliana*. Accordingly, the B-chelating role of anthocyanin might be increased as the level of B toxicity increased in *Arabidopsis thaliana*. Especially, directly applied B toxicity might induce more the B-chelating role of anthocyanin in plants. Coordinately, severe boron toxicity (3B(D) treatment) caused coordinated up-regulation of expressions of genes involved in anthocyanin synthesis and transport and related transcription factors, in WT *Arabidopsis thaliana*. This was validated by anthocyanin level under 3B(D) condition. In conclusion, anthocyanin biosynthesis might have rapid and transient mechanism of transcriptional regulatory complex in *Arabidopsis thaliana*. After this transient regulation of beginning and early steps, dramatic increases in transcription factors such as MYB75, MYB114, and anthocyanin transporter including *TT19* under severe B toxicity might primarily regulate late anthocyanin processes including vacuolar sequestration of anthocyanins, indicating compartmentalization of B-anthocyanin complexes in vacuoles in *Arabidopsis thaliana*.

References

Appelhagen I, Lu G-H, Huep G, Schmelzer E, Weisshaar B (2011). TRANSPARENT TESTA1 interacts with R2R3-MYB factors and affects early and late steps of flavonoid biosynthesis in the endothelium of Arabidopsis thaliana seeds. The Plant Journal for Cell and Molecular Biology 67: 406-419.

Aquea F, Federici F, Moscoso C, Vega A, Julian P et al. (2012). A molecular framework for the inhibition of Arabidopsis root growth in response to boron toxicity. Plant, Cell and Environment 35: 719-734.

Blokhina O, Virolainen E, Fagerstedt KV (2003). Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals Botany 91: 179-194.

Blount JW, Korth KL, Masoud SA, Rasmussen S, Lamb C et al. (2000). Altering expression of cinnamic acid 4-hydroxylase in transgenic plants provides evidence for a feedback loop at the entry point into the phenylpropanoid pathway. Plant Physiology 122: 107-116.

Bubna GA, Lima RB, Zanardo DY, Dos Santos WD, Ferrarese Mde L et al. (2011). Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (*Glycine max*). Journal of Plant Physiology 168: 1627-1633.

Chappell J, Hahlbrock K (1984). Transcription of plant defense genes in response to UV light or funga1 elicitor. Nature 311: 76-78.

Chomczynski P, Sacchi N (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162: 156-159.

Dixon RA, Paiva NL (1995). Stress-induced phenylpropanoid metabolism. The Plant Cell 7: 1085-1097.

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C et al. (2010). MYB transcription factors in *Arabidopsis*. Trends in Plant Science 15: 573-581.

Ehlting J, Buttner D, Wang Q, Douglas CJ, Somssich IE et al. (1999). Three 4-coumarate: coenzyme A ligases in *Arabidopsis thaliana* represent two evolutionarily divergent classes in angiosperms. The Plant Journal for Cell and Molecular Biology 19: 9-20.

Estévez L, Queizán M, Mosquera RA, Guidi L, Piccolo EL et al. (2021) Journal of Agricultural and Food Chemistry. doi: 10.1021/acs.jafc.0c06827

Gonzalez A, Zhao M, Leavitt JM, Lloyd AM (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in *Arabidopsis* seedlings. The Plant Journal for Cell and Molecular Biology 53: 814-827.

Gou JY, Felippes FF, Liu CJ, Weigel D, Wang JW (2011). Negative regulation of anthocyanin biosynthesis in *Arabidopsis* by a miR156-targeted SPL transcription factor. The Plant Cell 23: 1512-1522.

Hale KL, McGrath SP, Lombi E, Stack SM, Terry N et al. (2001). Molybdenum sequestration in Brassica: a role for anthocyanins? Plant Physiology 126: 1391-1402.

Hale KL, Tufan HA, Pickering IJ, George GN, Pilon M et al. (2002). Anthocyanins facilitate tungsten accumulation in Brassica. Physiologia Plantarum 116: 351-358.

Hinderer W, Seitz HU (1985). Chalcone synthase from cell suspension cultures of *Daucus carota* L. Archives of Biochemistry and Biophysics 240: 265-272.

Jones DH (1984). Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. Phytochemistry 23: 1349-1359.

Kayıhan DS, Kayıhan C, Çiftçi YÖ (2016). Excess boron responsive regulations of antioxidative mechanism at physio-biochemical and molecular levels in *Arabidopsis thaliana*. Plant Physiology and Biochemistry 109: 337-345.

Kayıhan C, Öz MT, Eyidoğan F, Yücel M, Öktem HA (2017). Physiological, biochemical, and transcriptomic responses to boron toxicity in leaf and root tissues of contrasting wheat cultivars. Plant Molecular Biology Reporter 35: 97-109.

Kayıhan DS, Kayıhan C, Çiftçi YÖ (2019). Regulation of boron toxicity responses via glutathione-dependent detoxifcation pathways at biochemical and molecular levels in *Arabidopsis thaliana*. Turkish Journal of Botany 43: 749-757.

Kitamura S, Shikazono N, Tanaka A (2004). *TRANSPARENT TESTA 19* is involved in the accumulation of both anthocyanins and proanthocyanidins in *Arabidopsis*. The Plant Journal 37: 104-14.

Koes R, Verweij W, Quattrocchio F (2005). Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. Trends in Plant Science 10: 236-42.

Landi M, Degl'Innocenti E, Pardossi A, Guidi L (2012). Antioxidant and photosynthetic responses in plants under boron toxicity: a review. American Journal of Agricultural and Biological Science 7: 255-270.

Landi M, Guidi L, Pardossi A, Tattini M, Gould KS (2014). Photoprotection by foliar anthocyanins mitigates effects of boron toxicity in sweet basil (*Ocimum basilicum*). Planta 240: 941-953.

Landi M, Tattini M, Gould KS (2015). Multiple functional roles of anthocyanins in plant-environment interactions. Environmental and Experimental Botany 119: 4-17.

Landi M (2015). Can anthocyanins be part of the metal homeostasis network in plant? American Journal of Agricultural and Biological Science 10: 170-177.

Landi M, Margaritopoulou T, Papadakis IE, Araniti F (2019). Boron toxicity in higher plants: An update. Planta 250: 1-22.

Lang D, Weiche B, Timmerhaus G, Richardt S, Riaño-Pachón DM et al. (2010). Genome-wide phylogenetic comparative analysis of plant transcriptional regulation: a timeline of loss, gain, expansion, and correlation with complexity. Genome Biology and Evolution 2: 488-503.

Mancinelli AL, Yang CPH, Lindquist P, Anderson OR, Rabino I (1975). Photocontrol of anthocyanin synthesis III. The action of streptomycin on the synthesis of chlorophyll and antyocyanin. Plant Physiology 55: 251-257.

Maruyama-Nakashita A, Nakamura Y, Yamaya T, Takahashi H (2004). A novel regulatory pathway of sulfate uptake in Arabidopsis roots: Implication of CRE1/WOL/AHK4-mediated cytokinindependent regulation. The Plant Journal 38: 779-789.

Maruyama-Nakashita A, Nakamura Y, Tohge T, Saito K, Takahashi H (2006). Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. The Plant Cell 18: 3235-3251.

Mengel K, Kirkby EA (2001). Principles of Plant Nutrition, 5th ed. Bern, Switzerland: International Potash Institute.

Miwa K, Fujiwara T (2011). Role of overexpressed BOR4, a boron exporter, in tolerance to high level of boron in shoots. Soil Science and Plant Nutrition 57: 558-565.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Nable RO (1988). Resistance to boron toxicity amongst several barley and wheat cultivars: a preliminary examination of the resistance mechanism. Plant and Soil 112: 45-57.

Nable RO, Banuelos GS, Paull JG (1997). Boron toxicity. Plant and Soil 193: 181-198

Nikiforova V, Gakière B, Kempa S, Adamik M, Willmitzer L et al. (2004). Towards dissecting nutrient metabolism in plants: a systems biology case study on sulfur metabolism. Journal of Experimental Botany 55: 1861-1870.

Öz MT, Yılmaz R, Eyidoğan F, Graaff L, Yücel M et al. (2009). Microarray analysis of late response to boron toxicity in barley (*Hordeum vulgare* L.) leaves. Turkish Journal of Agricultural and Forestry 33:191-202.

Papadakis IE (2016). The timeless contribution of rootstocks towards successful horticultural farming: from ancient times to the climate change era. American Journal of Agricultural and Biological Science 11:137-141.

Papadakis IE, Tsiantas PI, Tsaniklidis G, Landi M, Psychoyou M et al. (2018). Changes in sugar metabolism associated to stem bark thickening partially assist young tissues of Eriobotrya japonica seedlings under boron stress. Journal of Plant Physiology 231: 337-345.

Pardossi A, Romani M, Carmassi G, Guidi L, Landi M et al. (2015). Boron accumulation and tolerance in sweet basil (*Ocimum basilicum* L.) with green or purple leaves. Plant Soil 395: 375-389.

Petrussa E, Braidot E, Zancani M, Peresson C, Bertolini A et al.(2013). Plant flavonoids-biosynthesis, transport and involvement in stress responses. International Journal of Molecular Sciences 14: 14950-14973.

Poustka F, Irani NG, Feller A, Lu Y, Pourcel L et al. (2007). A trafficking pathway for anthocyanins overlaps with the endoplasmic reticulum-to-vacuole protein-sorting route in arabidopsis and contributes to the formation of vacuolar inclusions. Plant Physiology 145: 1323-1335.

Reid R (2007). Identifcation of boron transporter genes likely to be responsible for tolerance to boron toxicity in wheat and barley. Plant and Cell Physiology 48: 1673-1678.

Reid R, Hajes JE, Post A, Stangoulis JCR, Graham RD (2004). A critical analysis of the causes of boron toxicity in plants. Plant, Cell and Environment 25: 1405-1414.

Ryder TB, Cramer CL, Bell JN, Robbins MP, Dixon RA et al. (1984). Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of the phytoalexin defense response. Proceedings of the National Academy of Sciences of the United States of America 81: 5724-5728.

Schröder J, Schäfer E (1980). Radioiodinated antibodies, a tool in studies on the presence and role of inactive enzyme forms: regulation of chalcone synthase in parsley cell suspension cultures. Archives of Biochemistry and Biophysics 203: 800-808.

Shi, M-Z, Xie D-Y (2014). Biosynthesis and metabolic engineering ofanthocyanins in *Arabidopsis thaliana*. Recent Patents Biotechnology 8: 47-60.

Shields SE, Wingate VP, Lamb CJ (1982). Dual control of phenylalanine ammonia-lyase production and removal by its product cinnamic acid. European Journal of Biochemistry 123: 389-395.

Vernoux T, Wilson RC, Seeley KA, Reichheld JP, Muroy S et al. (2000). The root meristemless1/cadmium sensitive2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12: 97-109.

Wakuta S, Fujikawa T, Naito S, Takano J (2016). Tolerance to excessboron conditions acquired by stabilization of a BOR1 variant with weak polarity in *Arabidopsis*. Frontiers in Cell and Developmental Biology 4: 4.

Whitehead JM, Dixon RA (1983). Chalcone synthase from cell suspension cultures of *Phaseolus vulgaris*. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 747: 298-303.

Yamaguchi C, Khamsalath S, Takimoto Y, Suyama A, Mori Y et al. (2020). SLIM1 transcription factor promotes sulfate uptake and distribution to shoot, along with phytochelatin accumulation, under cadmium stress in *Arabidopsis thaliana*. Plants 9, 163.

Yıldırım K, Uylaş S (2016). Genome-wide transcriptome profiling of black poplar (*Populus nigra* L.) under boron toxicity revealed candidate genes responsible in boron uptake, transport and detoxification. Plant Physiology and Biochemistry 109: 146-155.